RAPID AND SENSITIVE DETECTION OF PATHOGENIC BACTERIA IN CHICKEN PRODUCTS BY SINGLE TUBE NESTED REAL-TIME PCR

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ABSTRACT

Salmonella spp. and *Campylobacter jejuni* are highly infectious and leading causes of human bacterial gastroenteritis throughout the whole world, as well as in Hawaii where the reported cases were approximately 300 and 750 each year, respectively. The diseases associated with these pathogens are a major cause of morbidity, which is a significant public health concern. In the United States, chicken is commonly contaminated by *Salmonella* spp. and *Campylobacter jejuni.* Traditional culture-based methods for their detection are time-consuming, cumbersome, and lacking in reliability. Thus, this study aimed to explore a molecular technique named single tube nested real-time polymerase chain reaction (STN-rtPCR) to overcome the drawbacks of culture-based methods and enable rapid detection of *Salmonella* spp. and *Campylobacter jejuni* in chicken products.

Initially, a single tube nested PCR (STN-PCR) assay was developed for the detection of *C. jejuni* in artificially contaminated ground chicken homogenate. Nested primers were designed based on the hippuricase (*hip*O) genes of *C. jejuni*. The annealing temperatures and concentrations of nested primers were optimized. The specificity of the established STN-PCR assay was evaluated with thirteen bacterial strains. The sensitivity of the assay was evaluated with a serial dilution of *C. jejuni* DNA and *C. jejuni* cells in artificially contaminated ground chicken homogenate. In addition, the efficacy of the STN-rtPCR assay was compared with standard culture-based methods and conventional rtPCR for identification of *C. jejuni* in artificially contaminated ground chicken homogenate at different enrichment time. As a result, the optimum annealing temperatures for the outer and inner primers were 65^{o}C and 55^{o}C, respectively. The concentrations of outer and inner primers were chosen as 0.1 pmol

and 40 pmol, respectively. No amplicon was generated using tested non-target bacterial strains as templates. The sensitivity was determined to be 10 *C. jejuni* DNA copies, which was 100 times more sensitive than conventional PCR with inner primers. Furthermore, this assay was able to detect as low as 36 CFU/ml of *C. jejuni* in artificially contaminated ground chicken homogenate without enrichment. Besides, after 24 h of enrichment, the ground chicken homogenate with an initial inoculum of 0.1 CFU/g of *C. jejuni* was identified correctly by STN-rtPCR, while it was not tested positive by both culture-based methods and conventional rtPCR until the sample had been enriched for 48 h. Moreover, single *C. jejuni* cells per gram ground chicken, that was tested positive by the culture-based methods after 48 h of enrichment, was identified correctly by STN-rtPCR after 6 h of enrichment.

Moreover, a multiplex STN-rtPCR assay was developed for concurrent detection of *Salmonella* spp. and *C. jejuni*. Nested primers for the detection of *Salmonella* spp. were designed to target the *invA* gene. The annealing temperatures and concentrations of *Salmonella* primers were optimized based on the amplification conditions of the STN-rtPCR assay for *C. jejuni* as described above. The sensitivity and efficacy of established multiplex STN-rtPCR assay were evaluated with pure DNA of *S.* Typhimurium and *C. jejuni*. The performance of the developed assay was demonstrated with the artificially contaminated chicken rinse. The results showed the established multiplex STN-rtPCR assay yielded expected amplicons of 226 bp and 173 bp for *Salmonella* spp. and *C. jejuni*, respectively, while no amplification products were observed with non-target bacteria. The detection sensitivity was determined to be 1×10^{-3} ng/μl of *Salmonella* and *C. jejuni* DNA, and 10^2 CFU/ml of *Salmonella* and *C. jejuni* in the chicken rinse. Additionally, the assay exhibited a

comparable efficiency for co-amplifying 10^7 to 10^2 CFU/ml of *Salmonella* and *C. jejuni* in chicken rinse.

In summary, the developed single tube nested real-time PCR assays displayed a promising approach for simultaneously detecting *Salmonella* spp. and *Campylobacter jejuni* in chicken products with reduced time. It showed advantages of rapidity, high sensitivity and specificity, and low riks of cross contamination due to its closed-tube format. Moreover, this study would provide valuable information to food testing institutions and food manufacturers, which is necessary for preventing the spread of diseases and reducing economic losses.

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Chapter 1

Introduction

Food contamination caused by pathogenic bacteria has attracted increasing attention worldwide. According to the Centers for Disease Control and Prevention, each year approximately 48 million Americans become sick due to the ingestion of contaminated food. About 128,000 are hospitalized and 3,000 die (Alves *et al*., 2016). Food contamination not only causes millions of dollars in losses on food manufacturers but also poses severe threats to human health. *Salmonella* and *Campylobacter jejuni* account for most of foodborne infections. With the use of culture-independent diagnostic tests, the incidences of *Campylobacter* and *Salmonella* infections in 2018 were determined to be 9,723 and 9,084, respectively (Tack *et al*., 2019). It was recorded that several multistate outbreaks associated with *Salmonella* infection were related to poultry products, raw seafood, pre-cut fruits, and dairy products (CDC, 2019). Meanwhile, increasing evidences have proven that most cases of foodborne illnesses caused by handing and consumption of undercooked poultry products were attributed to *C. jejuni* contamination (Geng *et al*., 2019; Pielaat *et al*., 2018). Therefore, specific and sensitive detection methods are urgently needed for these pathogenic bacteria.

Conventional culture-based detection methods involve enrichment in nutritious broth, isolation on selective agar, and identification based on morphological, biochemical, and/or serological characteristics. They often require several days to obtain results and do not always supply information rapidly enough to allow appropriate actions needed to protect the public. In particular, it has a high potential to generate false positive results due to non-selective enrichment or false negative results because of viable but nonculturable (VBNC) state of target

bacteria. Recent advances in molecular biology provide new strategies for microbiological analysis of food. The polymerase chain reaction (PCR), which amplifying the target DNA template with the implement of species-specific primers, is a promising molecular tool for identification of pathogenic bacteria with reduced time. It has the advantages of great efficiency, superior specificity, and high sensitivity. In addition, nested PCR is a variant of conventional PCR. It uses two pairs of primers (inner pair and outer pair) to amplify a particular DNA segment, which can remarkably enhance the sensitivity of molecular detection. However, potential cross contamination of amplicons during additional manipulation of amplification products might increase the risk of false positive results as well. Therefore, the single tube nested PCR assay has been introduced to overcome this drawback by integrating the two-tube nested PCR reactions into a single-tube reaction (Kemp *et al*., 1990). It dramatically reduces the cross-contamination and provides sensitivity equal to or higher than those of previous detection methods. Moreover, multiplex PCR that detects multiple pathogens at the same time can further strengthen the efficiency of detection by saving cost and time. Besides, with the cooperation of real-time PCR, the detection can be dramatically powered by effectively quantifying the target DNA with specificity and reliability (Espy *et al*., 2006; Mackay, 2007).

In this study, single tube nested real-time PCR (STN-rtPCR) was explored to detect foodborne pathogenic bacteria. A STN-rtPCR assay was developed to identify *Campylobacter jejuni*. Its performance was compared with standard culture methods and conventional real time PCR in detecting the pathogen in pure culture and ground chicken homogenate. Furthermore, a multiplex STN-rtPCR assay using TaqMan probes was established for concurrent detection of *Salmonella* and *C. jejuni*. The applicability of this assay was investigated with chicken rinse artificially inoculated with *Salmonella* Typhimurium and *C. jejuni*.

Chapter 2

Literature Review

2.1 Foodborne Disease

Food safety issues have always been a worldwide public health concern. Although modern science and technology have reached a remarkable level, foodborne diseases remain a major cause of morbidity and mortality and severely affect the public health in both developed and developing countries (Murray *et al*., 2012). Foodborne disease refers to a type of disease that has infectious or poisonous properties caused by various hazardous factors that enter the human body through food intake. The known factors are pathogenic bacteria, viruses, parasites, harmful toxins and chemicals (Scallan *et al*., 2011). It was estimated that 39% of foodborne illnesses, 64% of hospitalizations and 64% of deaths were due to pathogenic bacteria each year in the United State (Havelaar *et al*., 2015; Scallan *et al*., 2011).

Common symptoms associated with the foodborne disease are nausea, vomiting, diarrhea, abdominal pain, and cramps (CDC, 2018). More severe illnesses may cause neurological damages, kidney failure, hemolytic uremic syndrome (HUS), Guillan-Barré syndrome, or even death (Schnee and Petri, 2017). Children and pregnant women have more chance of suffering from these diseases because of their weak immune systems (Hoffmann *et al*., 2017). Additionally, certain families with low- and middle-income are particularly at risk due to lacking awareness of food poisoning, inadequate food processing, and poor conditions for food storage (Grace, 2015). The main concern is fresh produce which is commonly eaten raw and handled under unsanitary conditions. Fresh fruits and vegetables are of great probability to be crossed contaminated during the washing procedure (Alegbeleye *et al*., 2018; Rana *et al*., 2010). The

consumption of raw milk is considered a serious issue because of potential fecal contamination during milking (LeJeune *et al*., 2009). Raw meat, especially poultry products, is well-documented to be associated with *Salmonella* infections (Anderson *et al*., 2016). Though modern industries are equipped with sophisticated hygiene systems, more strict biosecurity measures are required to eliminate potential contamination and cross-contamination problems.

2.2 Foodborne Pathogens

The food safety problems caused by pathogenic bacteria are becoming increasingly prominent. Besides, the consumption of food contaminated by pathogenic bacteria plays a major role in the outbreak of foodborne diseases. In 2018, the Foodborne Disease Active Surveillance Network (FoodNet) reported 25,606 illnesses, 5,893 hospitalizations, and 120 deaths caused by common food-related pathogens (Tack *et al*., 2019). These data were derived from laboratorial culture-based and culture-independent diagnostic tests. Specifically, infections caused by *Campylobacter*, *Salmonella, Shigella,* Shiga toxin-producing *E. coli, Listeria, Yersinlla,* and *Vibrio* were investigated. It was reported that *Campylobacter* and *Salmonella* were the two leading pathogens, accounting for 9,723 and 9,084 infections, respectively (Tack *et al*., 2019).

2.2.1 *Campylobacter jejuni*

2.2.1.1 Characteristics of *Campylobacter jejuni*

The *Campylobacter* genus belongs to the family *Campylobacteraceae,* the order Campylobacterales, the class Epsilonproteobacteria, and the phylum Proteobacteria (Lastovica, 2014). The three species *C. jejuni*, *C. coli*, and *C. lari* are often recognized as the major source of campylobacteriosis, accounting for 95% of infections (Adedayo *et al*., 2008). *C. jejuni*,

especially, is the most common cause of human bacterial gastroenteritis in the United States (Marder *et al*., 2018).

C. jejuni is a Gram-negative, non-spore-forming bacterium. It is slim (0.5 to 8 μm long, 0.2 to 0.5 μm wide), curved, s-shape or spiral rods with a polar unsheathed flagellum at one or both ends (Penner, 1988). The flagellated ability facilitates a high degree of corkscrew-like motility of *C. jejuni* even in a viscous environment, which enables *C. jejuni* to colonize the intestinal mucosa (Lastovica, 2014). The major phenotypic characteristics of *C. jejuni* are hippurate hydrolysis positive, catalase and oxidase positive, and nitrate reduction to nitrite (Levin, 2007). Moreover, the hippuricase gene only exists in *C. jejuni.* Thus, the encoded enzyme is capable of transferring N-benzoylglycine (hippuric acid) into glycine and benzoic acid (Hani *et al*., 1995). Additionally, *C. jejuni* lacks the ability to display resistance to environmental stresses. Therefore, *C. jejuni* is fastidious and requires peculiarly growth conditions or unique growth media (Griffith and Park, 1990; Park, 2002). Furthermore, instead of oxidizing carbohydrates or degenerating complex substances, *C. jejuni* takes advantage of amino acids or tricarboxylic acid cycle intermediates as energy sources (Vandamme, 2000; Vliet and Ketley, 2001).

The genome of *C. jejuni* is generally 1.6 - 1.7 Mbp of AT-rich DNA with a low guanine-plus-cytosine (G+C) content of 28 to 38 mol% (Ketley, 1997; Lastovica, 2014). *C. jejuni* is microaerophilic with an optimal growth atmosphere of 5-10% oxygen, 3-10% carbon dioxide, and 85% nitrogen (Garénaux *et al*., 2008). In addition, thermophilic *C. jejuni* grows optimally between 37°C and 42°C. Nevertheless, due to the absence of cold shock protein genes with which bacteria are able to survive at low temperatures, *C. jejuni* does not grow below 30°C (Hazeleger *et al*., 1995; Hazeleger *et al*., 1998).

C. jejuni is capable of surviving in environments with a water activity (a_w) of 0.997, showing poor presence at $a_w = 0.987$ instead. Additionally, it is intolerant of osmotic stress, performing a sensitive reaction to a concentration of 2% sodium chloride (Doyle and Roman, 1982). *C. jejuni* appears to be absent at a pH below 4.9 or above 9.0, exhibiting an optimal growth at pH 6.5-7.5 (Doyle and Roman, 1981). Furthermore, the stationary phase cells of *C. jejuni* are uncommonly susceptible to mild heat and oxidative stress comparing to the exponential phase cells. This may be explained by the absence of σ-factor, RpoS, in regulating the reaction of bacteria in stationary phase to various types of stress (Kelly *et al*., 2001). Moreover, *C. jejuni* displays poor viability and culturability when it encounters unfavorable environments. Consequently, its morphological characteristics may change from a spiral shape into a coccoid form (Buck *et al*., 1983; Park, 2002).

2.2.1.2 Pathogenesis and Virulence of *Campylobacter jejuni*

The mechanisms for gastrointestinal diseases induced by *C. jejuni* have been theorized as flagella-mediated motility, intestinal cell adherence, invasion and colonization, and toxin production (Asakura *et al*., 2007; Blaser *et al*., 2008; Butzler *et al*., 1991; Dastia *et al*., 2010; Hu *et al*., 2008; Ketley, 1997; Levin, 2007; Park, 2002; Van Vliet *et al*., 2001; Young *et al*., 2007). After being ingested along with contaminated food or water, *C. jejuni* is able to pass through the small intestinal mucus layer with flagella and then colonize the distal ileum and colon (Hu *et al*., 2008). It is known that the adherence and damage of gut epithelial cells is the primary cause of diarrhea by *C. jejuni*. Van Deun *et al*. (2007) reported the ability of *C. jejuni* to invade into epithelial cells, which results in cellular inflammation, was associated with the production of cytolethal distending toxin (CDT), and further weakened the absorptive capacity of the intestine.

It was illustrated that the pathogenicity of *C. jejuni* was also related to its resistance to gastric acids and bile salts (Lin *et al*., 2003; Van Deun *et al*., 2007).

Motility and chemotaxis (response toward chemical stimuli) play an essential role in effective bacterial colonization of the small intestine. The expression of polar flagella is crucial for *C. jejuni* to attach on the intestinal epithelial cells and penetrate the mucous layer. The importance of flagella was validated by investigating the adherence ability of mutants without motile flagella, which resulted in a low level of penetration (Wassenaar *et al*., 1991). It was also confirmed that the chemotaxis was necessary for intestinal colonization. Takata *et al*. (1992) substantiated that non-chemotactic mutants of *C. jejuni* exhibited deficient movement in semisolid medium and loss of chemotactic behavior even with intact active flagella. Hugdahl *et al*. (1988) investigated the chemotactic behavior of *C. jejuni* toward several organic acids, carbohydrates, constituents of bile, and amino acids, which drew a conclusion that L-fucose along with bile and mucin was responsible for *C. jejuni* intestinal infections. Based on studies on flagellar system and its components of *C.jejuni*, it is clear that the motility and chemotaxis are of significance in its intestinal adherence, colonization, and invasion (Guerry, 2007; Nuijten *et al*., 1990).

The production of toxin also contributes to the pathogenesis of *C. jejuni*. The cytolethal distending toxin (CDT) is a well-documented *Campylobacter* toxin (Jain *et al*., 2008; Whitehouse *et al*., 1998). The active subunits of CDT were testified as *cdt*A, *cdt*B and *cdt*C by DNA sequencing. These genes play a role in arresting cell cycle at the G2/M phase, intriguing cellular distension, eventually resulting in cell death (Frisan *et al*., 2001; Ge *et al*., 2008; Yamasaki *et al*., 2006).

Other essential factors for the pathogenesis of *C. jejuni* are adhesion and invasion of the host intestinal cells (Wooldridge *et al*., 1997). It is believed that the damage and inflammation of epithelial cells due to the infection of *C. jejuni* by crossing the mucosal layer result in severe diarrhea (Van Vliet *et al*., 2001). In addition, the iron acquisition is one of the features of *C. jejuni* (Field *et al*., 1986). *C. jejuni* can take advantage of exogenous siderophores. Wai *et al*. (1995) isolated an iron storage protein, ferritin, from *Campylobacter*. A hypothesis was proposed that *C. jejuni* could utilize ferritin to adhere to the host cells and provide protection for itself in the circumstance of high O_2 concentration (Ketley, 1997).

2.2.1.3 Prevalence of *Campylobacter jejuni* in Foods and Sources of Infection

As a commensal bacterium, *Campylobacter jejuni* is widely found in the intestines of various animals, especially in poultry, wild birds and livestock. Since the infected animal has no apparent manifestation, *C. jejuni* can be carried for a long time and further induce pollution to the environment, food, and water. Overtly, *C. jejuni* is known as a leading foodborne pathogen causing an acute gastrointestinal disease called campylobacteriosis. In the United States, the incidence of *Campylobacter* infections was estimated by the Centers for Disease Control and Prevention (CDC) and the Foodborne Diseases Active Surveillance Network (FoodNet) to be 13.0 per 100,000 persons for all ages (CDC, 2010; CDC, 2011). The CDC'S Foodborne Disease Outbreak Surveillance System documented 209 foodborne *Campylobacter* outbreaks, associated with 2,234 illnesses from 2010 to 2015 (Geissler *et al*., 2017). In 2018, with the utilization of culture-independent diagnostic tests (CIDTs), it was confirmed that *Campylobacter* infections accounted for 9,723 illnesses, 1,811 hospitalizations, and 30 deaths. *Salmonella* infections accounted for 9,084 illnesses, 2,416 hospitalizations, and 36 deaths (Tack *et al*., 2019). Above all, Hawaii has the highest incidence of *C. jejuni* infection (Effler *et al*., 2001).

Common symptoms of *C. jejuni* infection include fever, nausea, stomach pain, vomiting, and severe bloody diarrhea. They usually last 2-5 days. If not treated in time, the infection may develop into a more serious disease, Guillain Barré Syndrome, which triggers damage to the human peripheral nervous system and results in weaken muscle, paralysis, and death (Allos *et al*., 1998; Nachamkin *et al*., 1998; Van Doorn *et al*., 2008). Epidemiological studies have testified an approximate does of 500 *C. jejuni* cells can trigger the disease.

The outbreak of *C. jejuni* infections is mostly relevant to consumption of raw or undercooked poultry products (Boes *et al*., 2005; Huang *et al*., 2018; Nielsen, 2002; Scott *et al*., 2015; Stanley and Jones, 2003; Tompkins *et al*., 2013), raw milk and by-products (Bianchini *et al*., 2004; Davis, 2016; Heuvelink *et al*., 2009; Schildt *et al*., 2006), and contaminated fresh produce and water (Horrocks *et al*., 2009; Jackson *et al*., 2014; Stanley *et al*., 1998). Additionally, contact with pets (CDC, 2018), along with touching live chicken or raw chicken meat remains a potential route for the transmission of *C. jejuni*. Avian species are regarded as a most common reservoir of *C. jejuni*, due to their average body temperature of 41.8 +/- 0.18 °C which is the optimal temperature for *C. jejuni* to grow (Blankenship *et al*., 1982). Sharp *et al*. (2016) have concluded that approximately 38% to 77% of *Campylobacter* infections were associated with chicken, which was followed by cattle with a range of 16% to 54%. Furthermore, during the slaughtering process, fecal matter might be transferred from the intestine to carcass and meat products (Humphrey *et al*., 2007). Hence, it is extremely important for commercial food establishments to monitor *C. jejuni* infections and to eradicate the contamination of pathogens through effective interventions (Lanier *et al*., 2018).

In summary, *C. jejuni* reveals extensive distribution in poultry and other food products, which gives rise to many outbreaks of campylobacteriosis globally. Considering the pathogenesis

and virulence of *C. jejuni* would benefit the development of prophylaxis, diagnosis, and chemotherapy of gastrointestinal infections caused by *C. jejuni*, and cultivate new approaches for rapidly detecting, sufficient controlling and even eliminating *C. jejuni* strains in food.

2.2.2 *Salmonella*

2.2.2.1 Characteristics

Salmonella is a Gram-negative, small rod shaped (approximately 0.6-1.0 x 2.0-4.0 pm), non-spore forming bacterium, which belongs to the family Enterobacteriaceae. It is facultatively anaerobic, has low nutritional requirements, and possesses peritrichous flagella. It was first identified by Theobald Smith *et al*. in 1855. They isolated a bacterial strain from intestines of pigs suffering from severe fever, and then named it after Smith's colleague, Dr. Daniel Elmer Salmon (Eng *et al*., 2015). Based on the analysis of 16s rRNA sequences, the genus *Salmonella* includes two species, *Salmonella enterica* and *Salmonella bongori.* Furthermore, according to their genetic relatedness and the application of multilocus enzyme electrophoresis assay, *S. enterica* is subdivided into six subspecies which are *S. enterica* subsp. *enterica*, *S. enterica* subsp. *salamae*, *S. enterica* subsp. *arizonae*, *S. enterica* subsp. *diarizonae*, *S. enterica* subsp. *houtenea* and *S. enterica* subsp. *indica* (Reeve *et al*., 1989). They are very similar in morphology, culture characteristics, biochemical properties, and antigenic structure, and highly pathogenic. Moreover, almost all serotypes can contaminate food and cause disease. Thus, they are zoonotic pathogens that commonly cause salmonellosis to humans and animals. It was reported that *Salmonella enterica* serovar Typhimurium (S. Typhimurium) and *S. enterica* serovar Enteritidis (S. Enteritidis) were widely distributed in nature and accounted for over 60 % of human illnesses caused by *Salmonella* (Finstad *et al*., 2012; Gosling *et al*., 2018).

Salmonella survives in a wide range of temperatures with the maximum and minimum growth temperatures of 49°C and 2°C, respectively. The optimal growth temperature for *Salmonella* is 35°C to 37°C. It is not resistant to heat and can be killed at 60°C for 15 min (Rowbury, 1995). The optimal growth pH for *Salmonella* occurs at neutral (7-7.5), but it is able to tolerate low pH of 3.8 and high pH of 9.5. It can be killed in 5 % carbolic acid within 5 min (Rowbury, 1995). Moreover, the biochemical reactions of *Salmonella* are of significance for its survival. *Salmonella* possesses the ability to ferment glucose, mannitol, maltose, and other monosaccharides, though it cannot ferment lactose and sucrose.

2.2.2.2 Virulence and Pathogenesis of *Salmonella*

Salmonella has a complex antigenic structure which is generally classified into three types: somatic (O), capsular (K) and flagellar (H) (Brenner *et al*., 2000). The heat-stable somatic O antigen is on the surface of *Salmonella*, which consists of lipopolysaccharide. The heat-labile H antigen is present in flagella, which is composed of protein and responsible for the activation of the immune response (Eng *et al*., 2015). The heat-sensitive K antigen has a subtype antigen called virulence (Vi) antigen, which is involved in the pathogenesis of *Salmonella*. The pathogenic *Salmonella* is invasive, possessing the ability to penetrate the epidermal layer of the small intestine and further invade the epidermis tissue through epidermal cells. With the presence of O antigen and Vi antigen, *Salmonella* can resist the phagocytosis of the host and release endotoxins (Bakowski *et al*., 2008; Hansen-Wester *et al*., 2002).

Generally, 10^5 cells of *Salmonella* can cause human illness, though a lower dose of *Salmonella* might trigger severe disease. It is believed that humans and animals infected with *Salmonella* can be asymptomatic or manifested as a clinically symptomatic lethal disease, which may aggravate morbidity or mortality, or reduce the productivity of animals. One pathogenic

mechanism of *Salmonella* infections is the invasion. *Salmonella* can invade the small intestine mucosa and adhere to the surface intestinal cells, which is mediated by the invading gene *inv* on its chromosome (dos *et al*., 2018; Jung *et al*., 2017;). Although *Salmonella* does not produce exotoxins, it can produce endotoxins with strong toxicity when the cell lysed (Dar *et al*., 2018). The release of endotoxins in the human body would increase body temperature and decrease the amount of white blood cells. These toxins are the main cause of chills, fever, and leukopenia.

The clinical symptoms of salmonellosis mainly include headache, nausea, abdominal pain, chill, vomiting, diarrhea and fever. Once invasive infection occurs, there might be severe complications that threaten the human life. It was documented that *Salmonella* could have a relentless impact on the bloodstream, brain, bone or joint, thus inducing bacteremia, meningitis, osteomyelitis or septic arthritis, respectively (Sánchez-Vargas *et al*., 2011).

2.2.2.3 Prevalence of *Salmonella* in Foods and Sources of Infection

Salmonella is one of the most important zoonotic pathogens. It is estimated that foodborne disease outbreaks caused by *Salmonella* account for 1.2 million illnesses, 23,000 hospitalizations, and 450 deaths each year in the U.S. (CDC, 2014; Scallan *et al*., 2011). Scharff (2018) reported the annual economic burden from foodborne illness in the United State, which estimated that the cost for *Salmonella* infections was the highest ($5.4 billion) among that of identified pathogens, due to a relatively long illness duration, a high number of hospitalizations and death rates. The main sources of *Salmonella* infections are (1) livestock and poultry with prenatal infection and post-mortem contamination; (2) milk from cows suffering from salmonellosis or contaminated by the outer environment after released; (3) egg products contaminated by the feces with *Salmonella*.

It was well-documented that raw poultry and poultry products contaminated by *Salmonella* remained a primary threat to public health and economic development (Cosby *et al*., 2015; Cox *et al*., 2011). There were outbreaks of *Salmonella* infection linked to chicken, raw turkey products, and ground beef in 2018 (CDC, 2018). It was confirmed that 92 individuals were infected by *Salmonella* in 29 states through consumption of raw or undercooked chicken products. Additionally, 265 *Salmonella* infections associated with chicken salad were reported from 8 states with 94 hospitalizations and one death. A recall of all chicken salad in grocery stores was issued to avoid further infections (CDC, 2018). Moreover, 90 cases of salmonellosis with 40 hospitalizations reported from 26 states were related to raw turkey products, and 120 cases with 33 hospitalizations were due to contaminated beef products. It was also reported that a variety of raw chicken products has triggered severe infections of *Salmonella*, which brought about a total of 129 illnesses with 25 hospitalizations among 32 states in 2019 (CDC, 2019).

The routes of egg contamination by *Salmonella* are: (i) stains which are present in the gut or feces of infected hens penetrate the eggshell during oviposition (Messens *et al*., 2005); and (ii) strains which are present in infected reproductive organs directly contaminate albumin, yolk, and eggshell membranes (Okamura *et al*., 2001). There were 45 cases of foodborne illnesses from raw eggs reported in 10 states.. It was indicated that the outbreak would be mostly due to handling and preparing fresh eggs improperly. In Hawaii, a survey was carried out to investigate the occurrence of *Salmonella* in locally produced eggs. As a result, 9.4 % of the 106 dozen eggs were tested shell positive for *Salmonella* (Ching-Lee *et al*., 1991). Suggestions are made by CDC to remind consumers to be aware of the potential *Salmonella* infections through eggs and handle the food carefully (CDC, 2018).

2.3 Detection of Foodborne Pathogens

An effective detection method is of significance in preventing pathogen-related foodborne illnesses as well as providing better protection for public health. It is required that the detection method can correctly identify a positive or negative sample and differentiate a wide kind of target pathogens from non-target organisms. Requirements also include reproducibility and repeatability, which indicate that the detection method allows superior performance across laboratories and generates the same results within a lab. Moreover, due to complex environmental factors, small changes have to be allowed for a more sensitive test (USDA-FSIS, 2017). However, the challenges for bacteria detection have been explained as the existence of low numbers of target cells in foods, interference of food ingredients, and inhibition by indigenous microflora. Common detection methods can be divided into conventional, immunological, and molecular methods.

2.3.1 Conventional Methods

Conventional microbial detection methods are culture-based. The identification of microorganisms by culturing is favored by regulatory agencies. These methods involve the assumption that an individual colony will be produced by a single bacterial cell once diluted cell suspensions are spread on an appropriate agar medium. Basic procedures are cultural enrichment, selective isolation, serological or biochemical screening, and strain confirmation. In order to recover injured cells in the sample, a pre-enrichment step is applied by using a non-selective broth. Pre-enrichment can also dilute inhibitors in the sample. Selective enrichment step takes advantage of a selective broth to increase the ratio of target pathogen to other microflora, which are suppressed by the same medium. Isolation of target pathogen is achieved by plating the

enriched culture on selective and differential agar. The appearance of a typical single colony or media color changes represents a positive result. Further serological or biochemical tests are applied to confirm the identity of strains isolated from the sample.

In general, the culture-based detection methods require 18-24 hours for enrichment and plating. According to the standard culture methods for *Salmonella* detection, it takes 16-20 hours to resuscitate injured cells or multiplicate the live cells in pre-enrichment, with another 18-48 hours for selective enrichment and 24-48 hours to obtain plating results (FDA, 2004). In comparison, it demands an average of 7 days to identify *Campylobacter* spp. in food samples, due to its microaerophilic and thermophilic properties. The standard methods for detection of *Campylobacter* spp. involve 22-50 hours of pre-enrichment based on sample types, 40-48 hours of selective enrichment, and 40-48 hours of biochemical tests (Biesta-Peters *et al*., 2018; Jacobs-Reitsma *et al*., 2018). Clearly, all conventional methods require multiple enrichment and characterization steps to obtain positive results. Therefore, they are time consuming and labor intensive.

2.3.2 Immunological Methods

Immunological methods are based on highly specific reactions between antigen and antibody. Based on the marker used to label antibodies and the measurements used to recognize the antigen-antibody complex, immunological methods can be classified as agglutination (Barrow, 1994), fluorescent immunoassay (Bokken *et al*., 2003), bioluminescence immunoassay (Hunter *et al*., 2010), chemiluminescence immunoassay (Zhang *et al*., 2014), radioimmunoassay (Hollinger *et al*., 1975), and enzyme-linked immunosorbent assay (Kumar *et al*., 2008). The enzyme-linked immunosorbent assay (ELISA) is a rapid and sensitive method for bacteria detection compared to the conventional method.

The ELISA method combines an enzyme with the specific antibody molecule to form an enzyme-labeled antibody conjugate. In the test, the sample contains target bacteria is first added to a solid-phase matrix which is coated with specific antibodies. Once the immobilized antibodies encounter a corresponding antigen, an antigen-antibody complex will form. After thorough washing, the enzyme-labeled antibodies are added and reacted with the antigen on target bacteria, thus creating a sandwich-like antibody-antigen-antibody complex. With a substrate added to the system, the enzyme reacts with it to form a colored reactant. Eventually, the results are measured based on change in the color density or quantified by an optical instrument.

Due to various modifications to the basic routine, ELISA formats have been divided into sandwich ELISA, indirect ELISA, direct ELISA, and competitive ELISA (Lequin, 2005). Valdivieso-Garcia *et al*., (2001) introduced a double monoclonal antibody sandwich ELISA for rapid detection of *Salmonella*, which has a detection limit of 10^4 CFU/ml after pre-enrichment in selective broth containing brain heart infusion, yeast extract, sodium hydrogen selenite, and sodium cholate. The same detection limit was obtained later by Kumar *et al*. (2008), who demonstrated that pre-enrichment-based ELISA was able to detect 10^4 CFU/ml *S.* Typhi with the buffered peptone water to be the most suitable enrichment medium. ELISA has also been applied to characterize human serum antibody response to *C. jejuni* infection, which illustrates the ability of this assay for diagnosing *C. jejuni* infection (Blaser and Duncan, 1984).

Other immunological methods take advantage of fluorescence or radioactive markers and have the characteristics of simplicity, rapidity, quantification capability, and applicability for a wide range of samples. Nonetheless, immunoassays usually have detection limits within a range of 10^3 to 10^5 CUF/ml, which are not adequate to identify pathogen contamination at a lower level

and might fail to forecast a potential risk. They are also costly due to the requirement of specific antibodies. Moreover, the interference of food ingredients may trigger false positive results due to the reactions between enzyme conjugated antibody and food samples.

2.3.3 Molecular Methods

The polymerase chain reaction (PCR) is a nucleic acid-based rapid detection method, which can generate results within 2 or 3 hours. It was invented by Kary Mullis in 1983 (Mullis *et al*., 1986). It has been extensively utilized in molecular biology and medical applications, such as clinical diagnosis, DNA cloning and sequencing, genomic manipulation, gene therapy, pathogen detection, and environmental analysis (Brown, 2016; Clark and Pazdernik, 2012; Clarridge *et al*., 2004; Kennedy *et al*., 2015; Park *et al*., 2011; Saengkerdsub *et al*., 2007). The principle of PCR is very similar to the DNA replication process occurring in a cell, which involves the amplification of the target DNA template with the implement of heat stable DNA polymerase, two specific oligonucleotide primers, dNTPs (dATP, dCTP, dGTP, dTTP), and $MgCl_2$ (Yilmaz *et al*., 2012). It is a process of repeating denaturation, primer annealing, and extension. During denaturation, double-stranded DNA is denatured into single strands by breaking hydrogen bonds between nucleotide bases at 92-96°C. During the annealing step, the temperature decreases, allowing the primers to bind to the complementary single strands of the DNA. Then the reaction is heated to 72°C during the extension step, and the DNA polymerase replicates the desired DNA strings along with primers. An optimal amount of time is required for each step to react effectively. Therefore, 25 to 35 cycles are usually considered for PCR, whereas excessive cycles may result in undesirable secondary products and inactivate the DNA polymerase (Lorenz, 2012). In summary, PCR demonstrates an exponential amplification of specific regions of target DNA.

PCR assay has been proved for effectively shortening the time spent on the enrichment of tested samples. It is more sensitive, specific, reproducible and precise than ELISA and culture-based methods. However, alive and viable target cells might not be distinguished by PCR assay. Moreover, food constituents have been proved to be a potential inhibitor of PCR resulting in decreased sensitivity or false-negative results (Schrader *et al*., 2012). Despite that, PCR does possess the potential of detecting the target organism without prior enrichment or concentration steps.

2.3.3.1 Conventional PCR

Due to the specifically conserved DNA sequences of pathogenic bacteria, a pair of primers can be designed to carry out PCR to achieve the purpose of detecting the bacteria in conventional PCR (Figure 1). Notably, this set of primers only anneals to specific type of target DNA. Amplicons are usually visualized as bands on electrophoresis gel by staining with fluorescent ethidium bromide.

Numerous conventional PCR assays have been developed for detection of *C. jejuni* and *Salmonella*. Levin (2007) listed several genes of *C. jejuni* used in PCR assays, including *cad*F, pVir, *cdt*, *Hip*O, and *VS1*. Bang *et al*. (2004) identified *C. jejuni* isolates from turkey by analyzing seven virulence and toxin genes including *cdt* and *cad*F. The results helped to characterize these putative pathogenic determinants detailly related to *C. jejuni* and benefited further investigation of *C. jejuni* infection in turkey production. Englen and Kelley (2000) developed a novel method for rapid DNA isolation. Genomic DNA was obtained by a mechanical disruption in the presence of a guanidine-based reagent. PCR was performed using primers based on the hippuricase gene (*Hip*O). Successfully, an expected 735 bp amplicons from *C. jejuni* appeared in the electrophoresis gel.

On the other hand, various primers have been designed for the detection of *Salmonella*, which include *invA* (Chiu and Ou, 1996; Cocolin *et al*., 1998; Hsu *et al*., 2011; Rahn *et al*., 1992), *oriC* (Espinoza-Medina *et al*., 2006; Kim *et al*., 2006; Yeh *et al*., 2002), *hilA* (Ziemer and Steadham, 2003), *ompC* (Kwang *et al*., 1996), and *rfc* (Luk *et al*., 1993). Kwang *et al*. (1996) illustrated that the PCR targeting the *ompC* gene was able to detect *Salmonella* in ground beef samples after 4-6 h of enrichment with an initial inoculum of 100 cells. Agarwal *et al*. (2002) applied PCR to analyze several food samples including fresh vegetables, poultry products, and milk products. They proved that a detection limit of an initial concentration of 10 cfu g^{-1} *Salmonella* in food was achievable after 6 h of enrichment. Nevertheless, Moganedi *et al*. (2007) optimized primer concentrations and cycling parameters to finally achieve a detection limit of 2.6×10^4 CFU/ml from pure culture and 26 CFU/ml from water samples after 6 h of non-selective enrichment.

While conventional PCR has been a well-established method for sensitive and specific detection of bacterial pathogens, it still has some limitations when it is applied to food samples. Most of all, the reproducibility may be affected by various food constituents, such as organic and inorganic substances, which was explained as (1) reaction and co-precipitation between substances and nucleic acids, (2) degradation or sequestration of nucleic acids, (3) change of chemical properties of nucleic acids, (4) incomplete melting of DNA, (5) decrease in specificity of primers, and (5) inactivation of DNA polymerases (Schrader *et al*., 2012). The DNA from background microflora in the sample may also inhibit the amplification by competing with target DNA (Schrader *et al*., 2012). However, the distinct mechanism of function of PCR inhibitors remains unknown (Haramoto *et al*., 2018). In addition, PCR cannot distinguish dead cells from live cells since DNA is quite stable after cells die, which may contribute to false-positive results

from dead target cells that may not attribute to pathogenicity. Moreover, the number of pathogenic microorganisms in food samples is relatively low. Therefore, an enrichment step is desired to increase the number of target bacteria to exceed the detection limit. Furthermore, reagents used for gel electrophoresis, such as ethidium bromide, are harmful to human health. Also, conclusions are made based on the size of amplicons, which indicates the conventional PCR lacks the capability of quantification (Paiva-Cavalcanti *et al*., 2010).

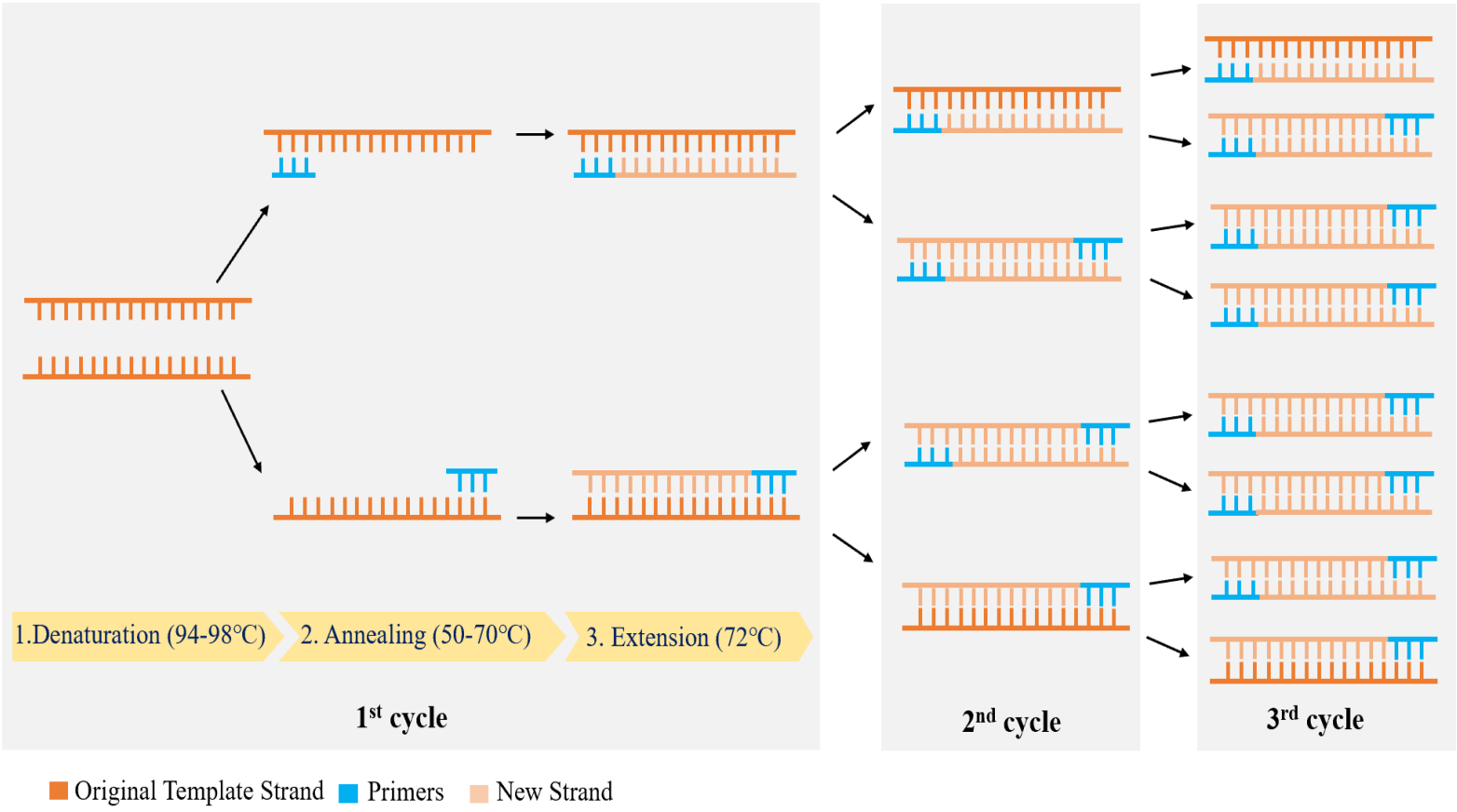

Figure 1. Schematic representation of exponential amplification of specific target by PCR.

2.3.3.2 Real-time PCR

Real-time PCR has revolutionized molecular diagnostic by effectively quantifying the target DNA copy number with specificity and reliability (Espy *et al*., 2006; Mackay, 2007). Compared with conventional PCR, real-time PCR does not involve gel electrophoresis to visualize the results, whereby it continuously and dynamically monitors the intensity of the fluorescent signal producing by a fluorophore (Law *et al*., 2015). The principle of quantification is the fluorescent data collected at the end of each cycle, which is directly proportional to the amount of PCR products. Detailly, a threshold is set based on the signal noise of the background. The number of cycles at which the detected signal intensity exceeds the threshold is named as Ct value. There is a linear relationship between the Ct value and the logarithm of the starting copy number of the template DNA. Therefore, as long as the Ct value of an unknown sample is obtained, target DNA in the sample can be quantified based on a standard curve made from known target DNA dilutions.

There are two types of fluorescence dyes, non-specific DNA binding dyes and target specific probes (Zhao *et al*., 2014). SYBR Green is the most commonly used non-specific fluorescence dyes (Law *et al*., 2015). SYBR Green binds to the minor groove of the double-strand DNA, emitting a fluorescent signal whose intensity represents the number of double-stranded DNA molecules. The dyes that are not incorporated into the DNA chain exhibit no fluorescent signal.

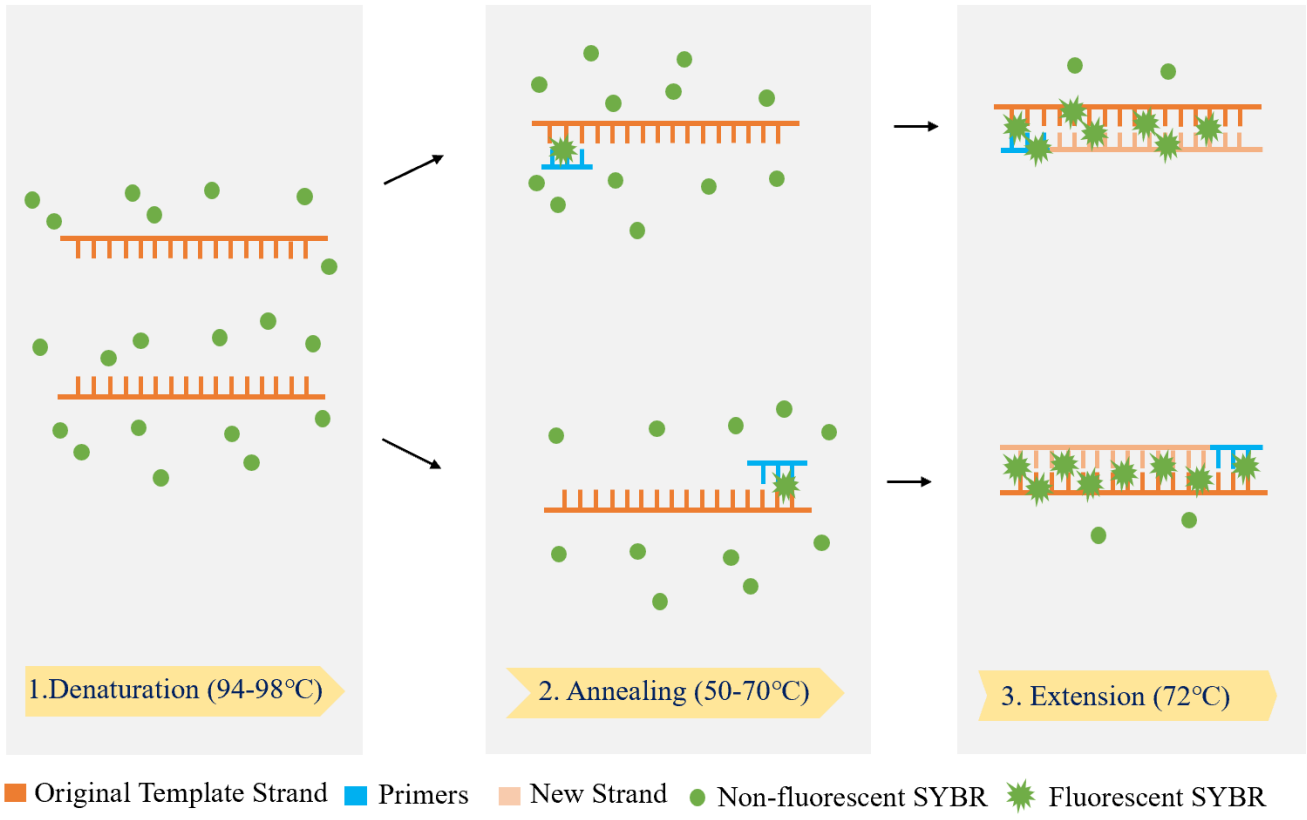

Figure 2. SYBR Green-based real-time PCR reaction.

Yang *et al*. (2004) presented a real-time PCR assay using SYBR Green for quantitative detection of *C. jejuni*. They demonstrated that their method could specifically identify *C. jejuni* from five non-target species and could be completed in 1 hour with a detection limit of 4×10^2 CFU/ml from bacterial culture. Nam *et al*., (2005a) developed a SYBR Green-based real-time PCR assay to detect *C. jejuni* in environmental samples from a naturally contaminated dairy farm. The detection limit was testified as approximately 10 CFU/ml and 10^3 CFU/ml of *C. jejuni* in broth and spiked lagoon water, respectively. After a 48-h enrichment, environmental samples with 10 CFU/ml of *C. jejuni* became identifiable. Oliveira *et al*. (2005) also applied the same method to detect *C. jejuni* on naturally contaminated chicken skin with a detection limit of 10 CFU per 10 g after 24 h of enrichment. However, Melero *et al*. (2011) reported quantification limits of 1 log CFU/ml and 2 log CFU/g after an enrichment of 48 hours for environmental and the meat samples, respectively. Ivanova *et al*. (2014) validated a FAST SYBR Green real-time PCR method for the identification of *C. jejuni* based on the amplification of the *hip*O gene. Furthermore, the established real-time PCR assay was applied to identify the presumptive colonies as belonging to *C. jejuni* from poultry samples after 48h enrichment and plating.

Additionally, Catarame *et al*. (2006) compared the performance of SYBR Green real-time PCR with a culture-based assay for *Salmonella* in Irish beef, which proved that the *Salmonella* was detected in meat samples by real-time PCR after 28 h of enrichment whereas the culture-based assay required 96 h of enrichment to gain results. Nam *et al*. (2005b) also testified the SYBR Green-based real-time PCR for detection of *Salmonella* in lagoon water, feed/silage, bedding soil, and bulk tank milk samples from a dairy farm. Results showed a minimum detection level of 10^3 to 10^4 CFU/ml of *Salmonella* in broth and 10 CFU/ml after 18 h enrichment. Bohaychuk *et al*. (2007) concluded the real-time PCR could detect 1 CFU of

Salmonella inoculated in beef carcass, pork carcass, chicken carcass rinse, lettuce, bovine fecal, equine fecal, and porcine fecal samples, and 2 CFU in chicken cecal contents, after 24 h of pre-enrichment and 24 h of selective enrichment. However, all inoculated samples gave negative results from the culture-based method. Alves *et al*. (2015) drew similar conclusions that 1 CFU/ml of *Salmonella* was detectable by real-time PCR after 18 h of enrichment in inoculated egg yolk, pizza, ground beef, pork sausage, chicken drumsticks, and cheese.

The advantage of SYBR Green dye is that it can monitor the amplification of any double-strand DNA sequences. However, since the fluorescent dye can bind to any double-strand DNA, it cannot perform multiplex PCR. It can also bind to non-specific double-strand DNA, such as primer dimer, potentially generating false positive signals. This problem can be solved by utilizing a specific hybridization probe which includes a reporter fluorescent dye at 5’ end and a quencher dye at 3’ end. The reporter dye is quenched by the quencher dye close to it, resulting in no fluorescent signal emitted. During PCR amplification, the DNA polymerase performs the extension of primer and can cleave the probe by its 5'-3' exonuclease activity. Consequently, the reporter dye is freed in the reaction system and emits the fluorescent signal. The accumulation of fluorescent signal represents the amplification of target DNA, which is explained as one DNA strand amplified for one fluorescent reporter molecule released (Law *et al*., 2015; Levin *et al*., 2004).

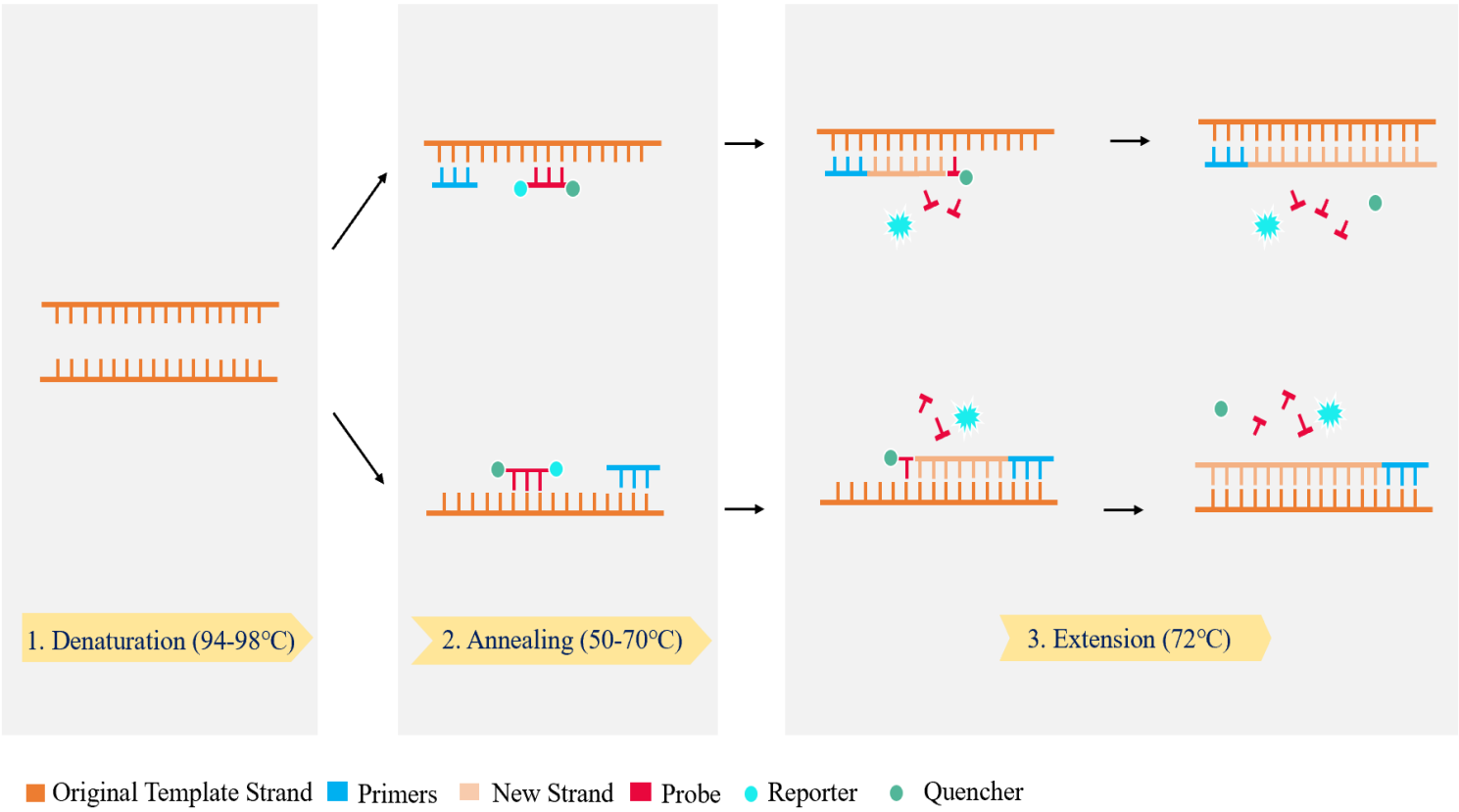

Figure 3. TaqMan probe based real-time PCR.

The TaqMan probe has been widely applied for more sensitive detection of pathogenic bacteria. Sails *et al*. (2003) reported a TaqMan based real-time PCR assay for successful detection of *C. jejuni* in raw poultry meat, raw shellfish, and milk samples after 48 h of enrichment. Liu *et al*. (2006) directly detected *C. jejuni* in food samples without enrichment by real-time PCR based on immunomagnetic-beads. Results indicated that a detection limit of approximately 10 CFU/ml in food samples within 8 h after 24 h enrichment. Suh *et al*. (2013) also applied a magnetic aptamer separation assay. The *C. jejuni* cells were first concentrated by magnetic aptamer followed by TaqMan probe-based real-time PCR, which resulted in a detection limit of 10 CFU/ml *C. jejuni* cells with a capture efficiency of lower than 5%.

Bohaychuk *et al*. (2006) developed TaqMan real-time PCR for identification of *Salmonella* in a wide variety of food and animal related matrices, including porcine feces and cecal contents, pork and beef carcasses, equine feces, and animal feed. Results showed a high sensitivity of this method compared with the culture-based method. A similar method was performed by Novinscak *et al*. (2007) for sensitive detection of *Salmonella*, targeting the *invA* gene, in composted biosolid. The results indicated that the method was efficient at detecting minimum 5.8 CFU of *Salmonella* after enrichment. Furthermore, Zhang *et al*. (2014) combined TaqMan real-time PCR with immunomagnetic separation (IMS) to detect healthy and heat-injured *Salmonella* Typhimurium in raw duck wings. Primers designed based on the *Sal*, *invA* and *ttr* genes were investigated. The separation step included a 30 min incubation for conjugating anti-*Salmonella* Dynabeads with *Salmonella* cells. It was testified that the *Sal* primers exhibited a lower detection limit of 10^3 CFU/ml after IMS than *invA* and *ttr* primers. *Salmonella* was successfully identified from raw duck wing samples after 7 h of enrichment. At the same time, Barbau-Piednoir *et al*. (2014) modified the real-time PCR assay with propidium monoazide

(PMA) to differentiate viable and dead *Salmonella* cells. With the technique, only viable cells would generate a fluorescent signal. However, it remained a problem that the signal from viable bacteria at low concentration might be suppressed by PMA. Moreover, a detection limit of 0.02 to 0.15 CFU/g of *Salmonella* in chili powder, soft cheese, fish, and tomatoes after 24 h enrichment in buffered peptone water was achieved by using TaqMan real-time PCR (Cheng *et al*., 2015).

The advantage of the TaqMan probe method is that its specificity is determined by specific primers and fluorescent probes. Therefore, it is not affected by non-specific amplification products and primer dimers, and its specificity is higher than that of fluorescent dyes. However, TaqMan also has some shortcomings: 1) enzyme activity may affect quantitative results because the hydrolysis of fluorescent emission groups utilizes the 5'-3' exonuclease activity of Taq polymerase; 2) the real-time PCR system is more susceptible to inhibitors existing in food samples; 3) the reporter and quencher on probe have to be made by specialized companies, which are expensive.

2.3.3.3 Nested PCR

Nested PCR is a variant of conventional PCR that uses two pairs of PCR primers (inner pair and outer pair) to amplify a DNA fragment. The first pair of PCR primers (outer) amplified fragments are similar to conventional PCR. The second pair of primers, called inner primers, binds inside the first PCR product such that the second PCR amplified fragment is shorter than the first amplicon. The advantage of nested PCR is that if the first amplification produces an erroneous fragment, the probability of primer pairing and amplification on the wrong fragment for the second time is extremely low. Therefore, the second set of primers is unlikely to amplify non-target fragments. This nested PCR amplification ensures that the second round of PCR

product has little or no contamination by non-specific amplification, which results in significantly higher specificity and sensitivity.

Winters *et al*. (1997) introduced a nested PCR for direct detection of *C. jejuni* in pure culture at a sensitivity of 10^2 CFU/ml. In their research, the outer primers, C-1 and C-4, were designed to amplify a specific region of *C. jejuni* DNA. For the nested reaction, the first round of PCR was performed by C-1 (forward) and C-4 (reverse) for 24 cycles. At this time, 1 μl of the PCR product from the first round of PCR reaction was removed and added to the second reaction as template. The second PCR assay was run with one of the outer reverse primers C-1 and an inner forward primer C-2 for 24 cycles. Moreover, Bang *et al*. (2002) developed a nested PCR assay with two different pairs of primers to detect *C. jejuni* in environmental samples from broiler farms. The two pairs of primers for *C. jejuni* were designed targeting *hip*O genes. These nested PCR assays can detect *C. jejuni* DNA at concentrations as low as 0.01-0.05 pg/PCR. In the detection of *Salmonella*, Rychlik *et al*. (1999) reported a detection limit of 10^5 CFU/g of feces without pre-enrichment and 10^2 CFU/g after pre-enrichment. However, in the same year, Waage *et al*. (1999) described a nested PCR for the detection of *Salmonella* in environmental water and meat samples. It was shown that as few as 10 CFU/100ml of *Salmonella* was identified in water after 1 h enrichment with background levels of up to 8700 heterotrophic organisms/ml water and less than an initial level of 10 CFU/g of *Salmonella* in meat samples enriched overnight. They proved that the nested PCR assay was applicable to detect low levels of *Salmonella* present in foods with a large number of background flora. Liu *et al*. (2002) further confirmed that nested PCR increased the sensitivity of detection by 100-fold, resulting in the detection of as few as four *Salmonella* cells in poultry samples after enriching for 18 h. Later, Saroj *et al*. (2008) investigated the efficiency and sensitivity of culture-based nested PCR for detection of

Salmonella in artificially contaminated sprouts, carrot, cucumber, and poultry meat samples. The sensitivity was testified as low as 4 CFU *Salmonella*/25 g of food samples after an enrichment of 6 hours.

Though nested PCR is effective in enhancing the sensitivity of conventional PCR, the potential for cross contamination of target DNA typically increases due to additional manipulation of amplicons. An established solution to reducing or even eliminating the cross contamination is to integrate the two-tube nested PCR reactions into a single-tube reaction. The single-tube nested PCR (STNP) was first described by Orou *et al*. (1995) in order to detect the mutations in human DNA, which highlighted that the well-designed primers/probe and optimization of the PCR protocol were essential for sensitive detection (Lin *et al*., 2010). The criteria for designing the primers are (1) the outer PCR products from the first round should be long enough to generate an appropriate inner amplicons, (2) the differences of annealing temperatures of inner primers and outer primers should be at least 10°C to allow for the separation of both rounds of PCR, and (3) both primer pairs should be highly specific to target DNA to increase the detection sensitivity (Llop *et al*., 2000). STNP assays have been successfully applied in the detection of human viruses, plant viruses, parasites, fungus, and bacterial toxins (Burkhardt *et al*., 2018; Dey *et al*., 2012; Feng *et al*., 2018; Hamim *et al*., 2018; Jang *et al*., 2015; Klemsdal and Elen, 2006; Llop *et al*., 2000; Saito *et al*., 2018).

There is little research described the detection of *Salmonella* by STNP, while there is no related report for *C. jejuni*. A one-tube nested PCR assay was established by Stankevicius *et al*. (2006) to detect 150 CFU/ml and 15 CFU/ml of *Salmonella* in swine faeces and saline solution, respectively. However, the mechanism behind this closed one-tube nested PCR was still separately performing two-round conventional PCR amplifications. Briefly, the reagents for the

first round PCR was added in the bottom of Eppendorf tubes, with the dried reagents for the second round PCR in the lid. Mineral oil was included as a vapor barrier. After the first round of amplification was completed, the tubes were inverted several times to dissolve the dried reagents in the lid, centrifuged briefly and subjected to the next amplification reaction. Nonetheless, Lin *et al*. (2010) introduced a strict single-tube nested PCR for detection of plant bacteria named *Candidatus* Liberibacter asiaticus. They designed outer primers with a higher annealing temperature of 65 °C and inner primers with a lower annealing temperature of 55 °C, which intended to prevent the interference from inner primers during the first round of PCR. In the STNP system, the amount of outer primers was lower than that of inner primers to allow efficient usage of outer amplification products. With TaqMan probe involving, the sensitivity of single-tube nested real-time PCR assay could reach single copies of target DNA. A similar assay was described by Day *et al*. (2012) for detection of pineapple mealybug wilt associated virus-2 resulting in a detection limit of 10 copies by TaqMan probe real-time PCR.

In summary, using sensitive single-tube nested PCR, pathogenic bacteria that cause many severe foodborne illnesses may be detected at an early stage enabling more efficient control strategies for safeguarding the food chain and protecting the public health.

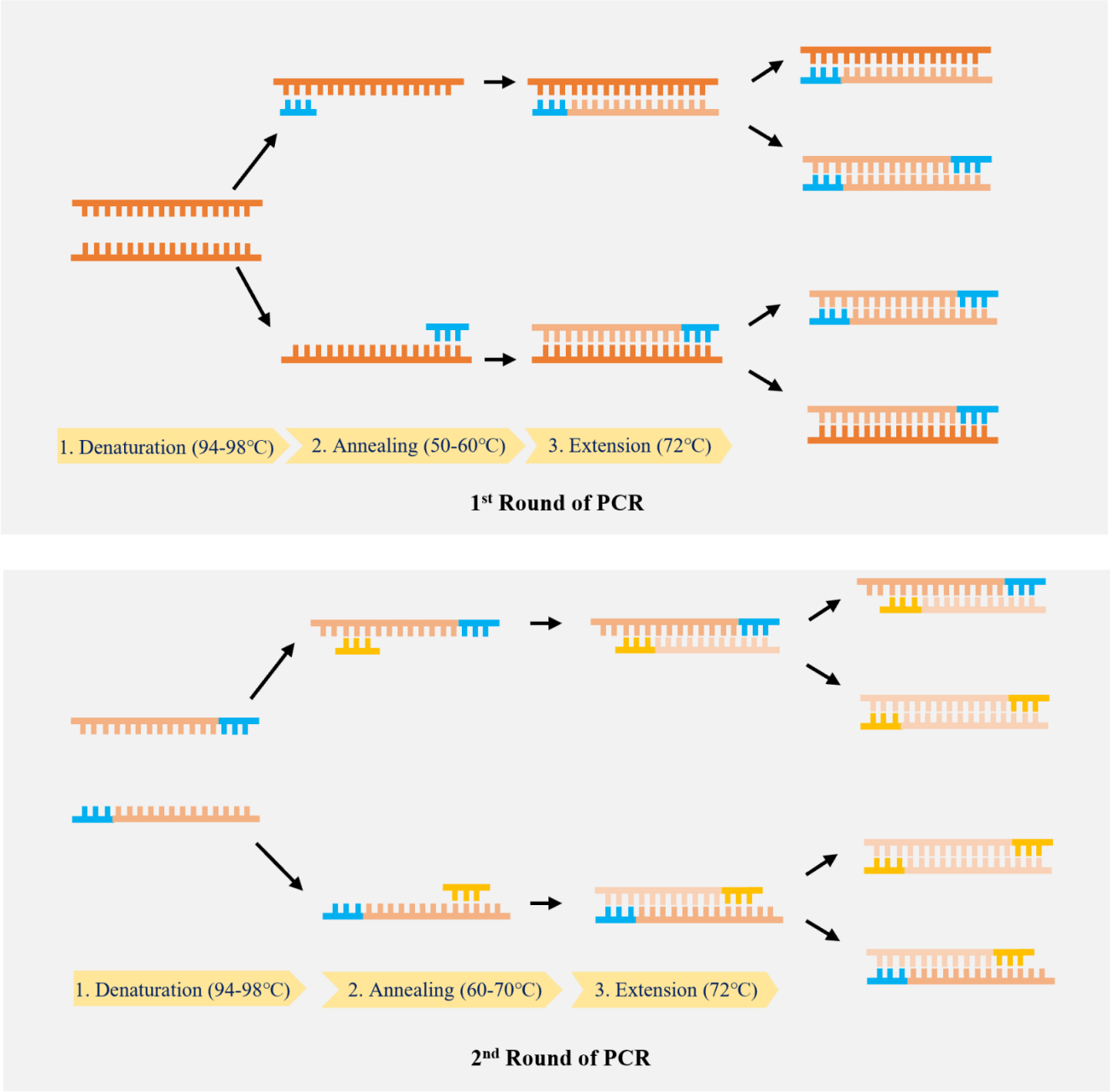

Figure 4. Nested PCR.

2.3.3.4 Multiplex PCR

Conventional PCR can only detect one pathogenic microorganism at a time. However, there potentially are many kinds of pathogens in food. Therefore, there has been a growing interest in simultaneously detecting multiple pathogenic bacteria. The multiplex PCR can meet the need. The principle of multiplex PCR is the same as that of conventional PCR except that multiple pairs of specific primers are added to the same reaction system. If templates complementary to each primer of pairs are present, a plurality of DNA fragments can be amplified simultaneously in the same reaction. The characteristics of multiplex PCR include high efficiency, simultaneous detection of multiple pathogens in the same reaction, and systemically analysis of multiple target genes. Multiplex PCR is suitable for analysis of a group of pathogenic bacteria causing the same symptoms or contaminating the same food. Detecting multiple pathogens simultaneously in the same reaction is economically friendly, which significantly reduces the detection time and reagents, and provides more accurate information for clinical or food safety testing. This method has also been widely used in various aspects of gene characterization, including amplification of long DNA fragments, detection of mutation deletion, polymorphism analysis, quantitative analysis, and genotyping.

It has to be noticed that the annealing temperature of each primer pairs should be optimized in order to operate harmoniously in a multiplex system. Furthermore, the sizes of each target should be distinctly distinguished in the electrophoresis gel. Otherwise, individual amplicons should be differentiable using TaqMan probes with different fluorescent dyes.

Multiplex PCR has been used in pathogen identification. Early in 2007, Wolffs *et al*. (2007) separated target organisms from background microflora by floating them in a discontinuous density gradient. Then, a real-time multiplex PCR assay with hybridization probes was

proceeded to detect *Campylobacter* spp. and *Salmonella* at a low level as 3.0×10^3 CFU/ml. Besides, Alves *et al*. (2012) indicated that the sensitivity of multiplex PCR for the detection of *Campylobacter* spp. and *Salmonella* spp. in spiked chicken meat rinses was 10^2 CFU/ml of *C. jejuni* after 24 h of selective enrichment and 1 CFU/ml of *Salmonella* Enteritidis after 24 h of nonselective enrichment. Alves *et al*. (2016) displayed a multiplex TaqMan probe-based real-time PCR assay including an internal amplification control for simultaneous detection of *Campylobacter* spp. and *Salmonella* spp. in chicken meat. They demonstrated that 0.1 ng of both target DNA could be detected in each reaction. A detection limit of 10^3 CFU of *Campylobacter* spp. and 10^6 CFU of *Salmonella* spp. per milliliter of artificially contaminated chicken meat rinse was confirmed without enrichment, while 1 CFU of each target per milliliter was identified after non-selective enrichment for 24 hours. Moreover, Al-Habsi *et al*. (2018) verified the applicability of real-time multiplex PCR for rapid identification of *C. jejuni* and *Salmonella* in goat fecal samples. Numerous studies have been performed for simultaneous detection of *Campylobacter* spp. and *Salmonella* spp. with other common foodborne pathogens, such as *E. coli* O157:H7, *Listeria* and *Shigella*, in water, chicken meat, poultry and other food samples (Barletta *et al*., 2013; Bonetta *et al*., 2016; Park *et al*., 2011; Santos *et al*., 2015; Skerniškytė *et al*., 2016; Van Lint *et al*., 2015).

From the aforementioned studies, it is evident that multiplex PCR has high specificity and sensitivity, and can simultaneously detect multiple pathogenic bacteria. It is more cost-, time-, and labor-effective than conventional PCR, exhibiting a good application prospect in clinical diagnosis and food testing. However, it requires careful system design and optimization to overcome potential unequal amplification of different target DNA in the same reaction.

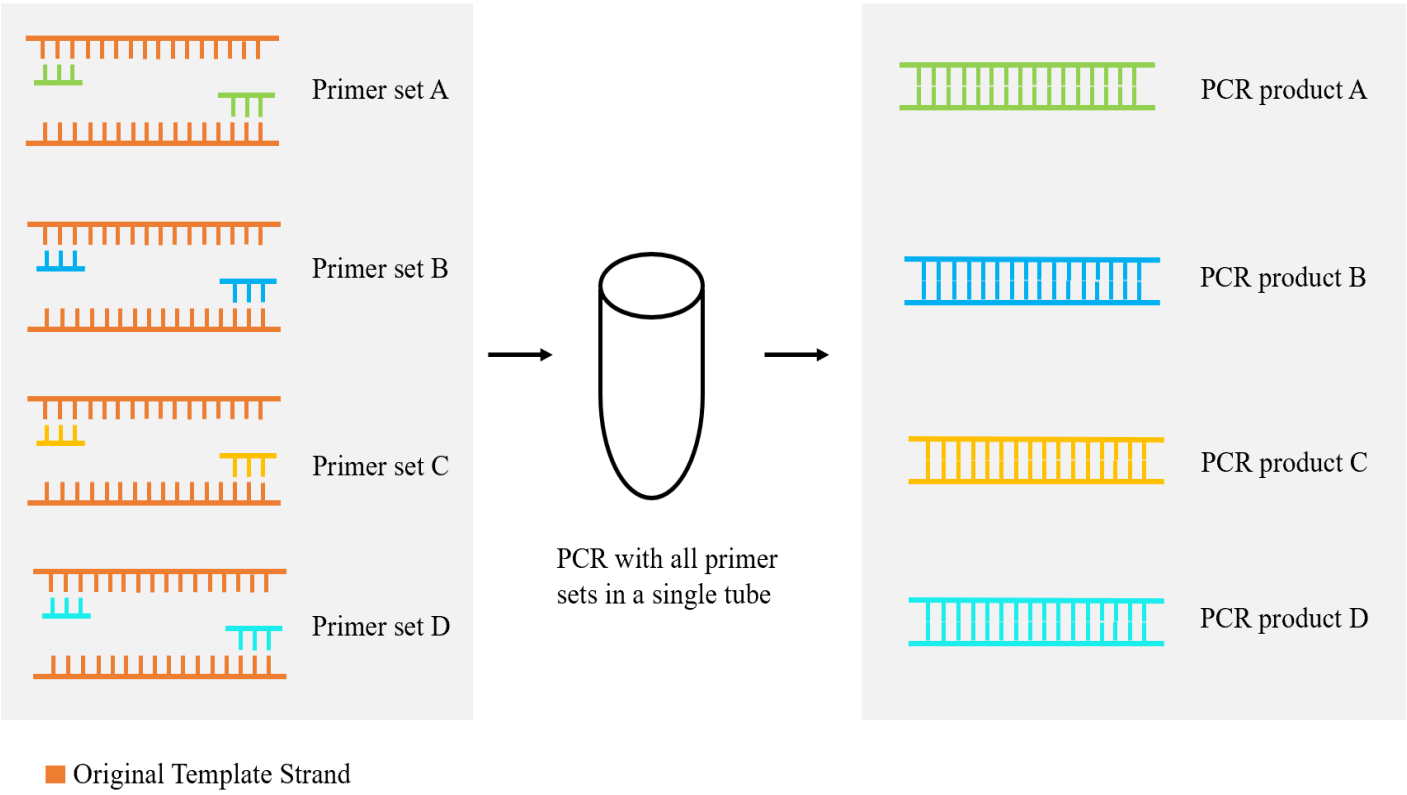

Figure 5. Multiplex PCR.

Chapter 3

Development of a Sensitive Single-Tube Nested PCR Assay for Rapid Detection of *Campylobacter jejuni* in Ground Chicken Homogenate

3.1 Introduction

Campylobacter jejuni is a gram-negative, rod-shaped, microaerophilic bacteria in the family *Campylobacteriaceae.* The *Campylobacter* species commonly associated with human or animal diseases are *C. jejuni*, *C. coli*, and *C. lari*, of which *C. jejuni* is the most frequent (80-90%) (Garin *et al*., 2012; Mezher *et al*., 2016). *C. jejuni* is considered to be a main cause of bacterial gastroenteritis worldwide, and it is estimated that there are approximately 1.3 million cases of human campylobacteriosis per year in the United States (Marder *et al*., 2018). In 2018, the Foodborne Diseases Active Surveillance Network (FoodNet) of the Centers for Diseases Control and Prevention identified *C. jejuni* as the leading pathogen resulting in 9,723 infections, 1,811 hospitalizations, and 30 deaths (Tack *et al*., 2019). *C. jejuni* is also the leading cause of foodborne illnesses in Hawaii, where the reported number is about 750 cases each year.

The most common symptoms of *C. jejuni* infection include bloody diarrhea, abdominal pain, fever and vomiting, and autoimmune neurological disorder such as Guillain-Barre syndrome, a debilitating and sometimes fatal paralysis (Blaser *et al*., 2008; Taboada *et al*., 2007). Studies have shown that as few as 500 *C. jejuni* cells can cause disease in human (Park *et al*., 2002). Moreover, there is a remarkably high incidence of *C. jejuni* infections associated with undercooked chicken, raw milk products, and untreated water (Havelaar *et al*., 2007; Zhao *et al*., 2001). The US Department of Agriculture (USDA) predicted that the national prevalence of *Campylobacter* was 21.70% in chicken parts, among which chicken neck (54.55%) and giblets (43.86%) posted the highest rates of contamination (USDA, 2012). Furthermore, according to the

National Antimicrobial Resistance Monitoring System (NARMS), *Campylobacter* occurred in 33% of raw chicken collected from retailers in 2014 (Marder *et al*., 2018). Currently, neither strict biosecurity measures on farms nor advanced hygienic systems in slaughterhouses have proven sufficient to prevent poultry meat from being contaminated by *C. jejuni*. All these factors underscore the need for a sensitive and specific method for rapid detection of *C. jejuni* in food.

Numerous methods have been developed for the identification of *C. jejuni*, including culture-based assay, most probable number (MPN) assay, enzyme linked immunosorbent assay (ELISA), polymerase chain reaction (PCR), and real-time PCR (Colles and Maiden, 2012; Gosselin-Théberge *et al*., 2016; Granato *et al*., 2010; Kulkarni *et al*., 2002; Maher *et al*., 2003; Man, 2011; Van Camp *et al*., 1993). Traditional culture-based methods are the gold standard used by the Food and Drug Administration (FDA) and USDA in food microbiological testing. However, the results are influenced by enrichment media and culture methods. Moreover, the procedures are time-consuming and laborious. The identification of a suspected *C. jejuni* infection takes 4 to 6 days due to a probably low number of the bacteria in samples. The MPN assay is also labor-intensive. Although this method can detect low level of target bacteria in a sample containing a large number of non-target organisms the method requires a variety of cumbersome biochemical tests to identify the target.

In the past few decades, molecular techniques such as PCR have been successfully applied to the detection of *C. jejuni*. PCR is more rapid and sensitive than culture-based assay. Numerous PCR assays have been developed for rapid detection of *Campylobacter* species (Kulkarni *et al*., 2002; Maher *et al*., 2003). The real-time PCR is an advancement of the conventional PCR technique, which is capable of quantifying foodborne pathogens (Debretsion *et al*., 2007; Pacholewicz *et al*., 2019; Zhang *et al*., 2018). Nevertheless, highly sensitive real-time PCR

requires expensive instruments and reagents which are an excessive demand for many laboratories.

Another highly sensitive and specific molecular technique is nested PCR, which involves a second amplification of products from the first round of PCR (Bang *et al*., 2002; Winters *et al*., 1997). However, due to the addition of the second round of PCR, there is a possibility of cross-contamination of target DNA among samples when transferring amplicons from the first reaction tube to the second reaction. Moreover, the procedures are complex, which double the time compared to conventional PCR. An alternative to eliminating the potential of falsely positive results is conducting nested PCR in a single closed tube (Kemp *et al*., 1990; Orou *et al*., 1995; McManus and Jones, 1995; Niepold and Schöber-Butin, 1997). The single tube nested PCR (STN-PCR) is carried out by designing outer primers and inner primers with different annealing temperatures to separate two rounds of PCR in a one-step procedure in a single tube. It dramatically reduces the risk of amplicon cross-contamination and provides sensitivity levels equal to or even higher than those of nested PCR with the use of less time and reagents. Numerous studies have reported successful application of STN-PCR to detect clinical diseases caused by viruses, plant viruses, parasites, fungi, bacterial toxins and nut allergens (Bertolini *et al*., 2003; Burkhardt *et al*., 2018; Costa *et al*., 2013a; Costa *et al*., 2013b; Dey *et al*., 2012; Feng *et al*., 2018; Hamim *et al*., 2018; Jang *et al*., 2015; Klemsdal and Elen, 2006; Llop *et al*., 2000; Saito *et al*., 2018).

This study aimed to develop a sensitive STN-PCR assay for the rapid detection of *C. jejuni* in artificially contaminated ground chicken homogenate. The specificity of established STN-PCR assay was investigated, and the sensitivity was compared with conventional PCR for the detection of *C. jejuni* in both pure cultures and ground chicken homogenate. Furthermore, *C.*

jejuni in ground chicken homogenate was quantified by using single-tube nested real-time PCR (STN-rtPCR). The performance of STN-rtPCR for sensitive detection of low levels of *C. jejuni* in enriched artificially contaminated ground chicken homogenate was compared with that of a standard culture-based method and a real-time PCR (rtPCR) assay.

3.2 Materials and Methods

3.2.1 Bacterial strains and culture conditions

This study was performed using three *C. jejuni* strains (ATCC 11168, Penn 4, and Penn 19), two *C. coli* strains (clinical and Penn 5), and one *C. lari* strain. Also, seven non-*Campylobacter* bacterial strains were *Proteus vulgaris*, *Bacillus cereus*, *Staphylococcus aureus*, *Shigella flexneri*, *Listeria monocytogenes*, *Escherichia coli* O157:H7, and *Salmonella* Typhimurium. These strains were obtained from Food Microbiology Lab collections at the University of Hawaii at Manoa. All strains were stored in 50 % glycerol at -80°C before use. All *Campylobacter* strains were cultivated in Muller Hinton broth (Becton Dickinson, Maryland, USA) at 42°C for 48 h in microaerophilic GasPak™ EZ Campy pouches (Becton Dickinson). Non-*Campylobacter* bacterial strains were aerobically grown in trypticase soy broth (Becton Dickinson) at 37°C for 24 h.

3.2.2 DNA extraction

The DNeasy Blood & Tissue Kit (QIAGEN, Germany) and PrepMan Ultra reagent (Applied Biosystems, UK) were used for DNA extraction. Total DNA was extracted from *C. jejuni* ATCC 11168 culture using DNeasy Blood & Tissue Kit according to the instructions of the manufacturer for Gram-negative bacteria. The final DNA concentration was measured using the NanoDrop2000 spectrophotometer (Thermo Scientific, Waltham, USA). The DNA concentration

was converted to the copy number based on the estimated *C. jejuni* chromosome of 1700 kb from GenBank. The calculation formula used was: Number of copies = (amount of DNA template [ng] × 6.022 × 10^{23} number/mole) ÷ (length of the template [bp] × 1 × 10^{9} ng/g × 650 g/mole of bp). The purified DNA was ten-fold serially diluted in DNase free water to final concentrations of 4.1 × 10^{6} copies/µl to 4.1 × 10^{1} copies/µl and stored at -20°C until use.

Total DNA from other twelve bacterial strains was extracted by using PrepMan Ultra reagent. Briefly, 0.1 ml of bacterial culture was transferred to a 1.5 ml microcentrifuge tube and centrifuge at 13,200 rpm for 1 min. Cell pellets obtained were resuspended in 0.1 ml PrepMan Ultra reagent and mixed by pipetting. The mixture was placed in boiling water for 10 min. After centrifugation at 13,200 rpm for 2 min, the supernatant was used as template DNA in PCR assays.

3.2.3 Primers design

Two sets of primers were designed based on the hippuricase (*hipO)* gene of *C. jejuni* using the Primer3 software. The criteria for inner primers were shown as follows: GC% ≥ 40-50, Tm = 55°C ± 2, primer length = 16-22 bp, and amplicon size of 150-300 bp. The same criteria were also applied to design the outer primers, except that a higher annealing temperature (at least 10°C higher than inner primers) and longer amplicon size (300-500 bp) were considered. The AmplifX software (http://crn2m.univ-mrs.fr/pub/amplifx-dist) was used to locate primers within the target gene and analyze the quality of primers to select the best inner and outer primers sets with the least possibility of forming self/cross dimers. Primers were also validated in silico against all microbial DNA sequences in NCBI databases (https://www.ncbi.nlm.nih.gov/).

3.2.4 Development of STN-PCR assay

Gradient PCR was conducted in a 48-well thermal cycler (MJ MINI PCR Thermal Cycler, Bio-Rad Laboratories, USA) to investigate the optimum annealing temperatures for the outer and inner primers at which both rounds of PCR could be separated. The annealing temperatures were set from 50°C to 65°C for inner primers and 60°C to 70°C for outer primers. PCR experiments were carried out in a volume of 25 µl containing 1 µl *C. jejuni* DNA, 10.5 µl nuclease-free water, 12.5 µl GoTaq ® Hot Start Colorless Master Mix (Promega), and 1 µl of 1 nM inner or outer primer pairs. The negative control was included by replacing the DNA with 1 µl of nuclease-free water. The PCR conditions were 4 min at 95°C, followed by 35 cycles of 45 s at 95°C, 45 s at various annealing temperatures, extension at 72°C for 45 s, and a final extension at 72°C for 5 min. Following the amplification, PCR products were analyzed by electrophoresis in 1.8% of agarose gels in 1× TAE buffer. The gels were visualized by UV light after being stained with GelGreen Nucleic Acid Stain (Biotium).

After the annealing temperatures have been optimized, the concentrations of inner and outer primers were investigated. The amounts of primers were tested on 0.05, 0.1, 0.5 or 1.0 pmol for the outer primers, and 5, 10, 20 or 40 pmol for inner primers in a volume of 25 µl mixture. The PCR conditions were 4 min at 95°C, followed by 20 cycles of 45 s at 95°C, 45 s at 65°C, extension at 72°C for 45 s, and then 30 cycles of 45 s at 95°C, 45 s at 55°C, extension at 72°C for 45 s followed by final extension at 72°C for 5 min. This PCR protocol was determined to be the most effective for the STN-PCR assay. Following the amplification, PCR products were analyzed as described above.

3.2.5 Specificity of the STN-PCR assay

Genomic DNA extracts of 13 bacterial strains, including three *C. jejuni* strains, two *C. coli* strains, one *C. lari* strain, and seven non-*Campylobacter* strains, were tested as templates in the developed STN-PCR assay to evaluate the specificity and reliability of the designed primers. The negative control was nuclease-free water.

3.2.6 Comparison of the sensitivity of conventional PCR and the STN-PCR

A serial dilutions of *C. jejuni* DNA ranging from 4.1×10^6 copies/µl to 4.1×10^1 copies/µl were used as templates to compare the sensitivity of STN-PCR and conventional PCR with the inner primers. For the conventional PCR, the concentrations of inner primers were 40 pmol; the amplification parameters were 95°C for 4 min, followed by 30 cycles of 45 s at 95°C, 45 s at 55°C, and 72°C for 45 s, and extension at 72°C for 5 min. For STN-PCR, 0.1 pmol outer primers and 40 pmol inner primers were used in the optimized PCR conditions. Water was used as negative control in all experiments, and PCR products were analyzed as described above.

3.2.7 Preparation of artificially contaminated ground chicken homogenate

Fresh ground chicken was purchased from a local supermarket. Twenty grams of ground chicken was placed into a sterile stomacher bag, to which 180 ml of 0.1 % peptone water was added. The mixture was homogenized at 260 rpm for 1 min in a stomacher blender (Seward, Stomacher 400 Circulator, England). Nine milliliter aliquots of ground chicken homogenate were distributed to sterile tubes (Falcon). *C. jejuni* pure culture was serially diluted with the ground chicken homogenate to achieve final concentrations ranging from 3.6×10^6 CFU/ml to 3.6×10^1 CFU/ml. An uninoculated control was prepared by thoroughly mixing 9 ml of ground chicken homogenate with 1 ml of 0.1 % peptone water. In addition, the uninoculated control was serially

diluted in peptone water and spread on plate count agar followed by incubating at 37°C for 24 h to enumerate natural flora in ground chicken homogenate.

Total DNA from the artificially contaminated ground chicken homogenate was extracted by using PrepMan Ultra reagent. Briefly, 10 ml homogenate mixtures were centrifuged at 2550 rpm for 3 min to pellet meat debris. The supernatant was then transferred to a new tube and centrifuged at 6,000 rpm for 20 min. The pellet was resuspended in 200 µl PrepMan Ultra reagent. The mixture was transferred to a new 1.5 ml microcentrifuge tube and placed in boiling water for 10 min followed by cooling at room temperature. After centrifugation at 13,200 rpm for 2 min, the supernatant was used as template DNA in the STN-PCR and conventional PCR assays described above.

3.2.8 Quantification of *C. jejuni* in ground chicken homogenate by STN-rtPCR

To quantitatively compare the sensitivity of conventional PCR and STN- PCR, real-time PCR was performed using the IQ5 System (Bio-Rad, CA, USA). DNA extracts from artificially contaminated ground chicken homogenate samples were used as templates. For conventional rtPCR, 40 pmol of inner primers, 12.5 µl of SYBR Green PCR Master Mix (Applied Biosystems, UK), 1 µl of DNA template were mixed with RNase free water to a final volume of 25 µl. The conditions were 4 min at 95°C, followed by 30 cycles of 45 s at 95°C, 45 s at 55°C, and 72°C for 45 s, and extension at 72°C for 5 min. For STN-rtPCR, 0.1 pmol outer primers, 40 pmol inner primers, 12.5 µl of SYBR Green PCR Master Mix, and 1 µl of DNA template were mixed with RNase free water to a final volume of 25 µl. The amplification was carried out with the established STN-PCR conditions. Both DNA extracted from uninoculated ground chicken homogenate and water were also run in triplicate as controls in the assays to ensure there was no DNA cross-contamination.

Fluorescence data were collected at the end of each extension step of the inner primer pairs. The Ct value (the cycles number at which fluorescence signal exceeds the fluorescence threshold), Ct mean and standard deviation of the triplicates were calculated for each *C. jejuni* dilution (Alves *et al*., 2016). Standard curve with equation and R-squared value were generated by plotting the Ct mean from each dilution versus Log cell concentration. The following formula was used to calculate the efficiency with slope of the line:

Efficiency (%) = $(10^{(-1/slope)}-1) *100$

3.2.9 Comparison of culture-based method, conventional rtPCR, and STN-rtPCR in detecting low levels of *C. jejuni* in ground chicken homogenate

Artificially contaminated ground chicken homogenates for enrichment were prepared following the Bacteriological Analytical Manual specified by FDA. Briefly, twenty grams of ground chicken were weighed in a sterilized stomach bag and homogenized with 180 ml of Bolton Broth (Merck KGaA, Germany) with supplement (Sigma-Aldrich, USA) and lysed horse blood (Quad Five, USA) at 260 rpm for 1 min. One milliliter of *C. jejuni* culture was serially diluted with the ground chicken homogenate. Ninety milliliters of the homogenate were inoculated with 10 ml of *C. jejuni*-spiked homogenate in a screw-capped 120 ml glass bottles to achieve various concentrations of *ca* 10^{-1} to 10^{3} CFU/g. Accurate inoculation levels were determined by plate counting on Muller Hinton agar. An additional 100 ml of uninoculated ground chicken homogenate was used as a negative control. The spiked homogenate samples were incubated in GasPak™ EZ box with GasPak™ EZ Campy pouch at 37^{o}C on an orbital shaker set at 200 rpm for 4 h, and then incubated at 42^{o}C for 24 h with shaking and 24 h without shaking.

At 0, 2, 4, 6, 8, 10, 12, 24 and 48 h during enrichment, 10 ml homogenate from each sample was aseptically transferred to a 15 ml tube, from which 0.1 ml was spread on modified Campylobacter Blood-Free agar (mCCDA) (Merck KGaA, Germany) with supplement (Sigma-Aldrich, USA) and incubated at 42°C for 48h under microaerophilic condition. Occurrence of small, shiny, round, and gray colonies with smooth edges on mCCDA was considered to be a positive result. Three suspected colonies from each sample were purified and directly used in the STN-PCR assay to confirm its identity. The STN-PCR products of non-*C. jejuni* colonies were further sequenced and then identified in NCBI databases.

The total DNA of collected homogenate was extracted as described above and then used in the STN-rtPCR and conventional rtPCR assays. The performance of three methods was evaluated by determining the shortest enrichment time needed to detect low levels of *C. jejuni* in ground chicken homogenate. Experiments were replicated three separate times.

3.3 Results

3.3.1 Primers designed for single-tube nested PCR

Primers shown in Figure 6 were designed using Primer3 software. The lengths of outer primers were 16 bp and 21 bp for forward primer (Outer-F) and reverse primer (Outer-R), respectively. The lengths of inner primers were 19 bp and 19 bp for forward primer (Inner-F) and reverse primer (Inner-R), respectively. The amplification product for the outer primer is 425 bp, and the inner primer is 226 bp. All four primers were tested via BLAST against all available microbial DNA sequences in NCBI databases and confirmed to be specific for *C. jejuni*. No primer self/cross dimers or non-specific amplification products were produced, and both outer

and inner primers produced products of expected sizes when individually amplified by PCR at their optimum annealing temperature.

Figure 6. Sequences of inner and outer primers used for single-tube nested PCR assay targeting the *hip*O gene of *C. jejuni*.

3.3.2 Optimization of annealing temperatures and primer concentrations in STN-PCR

To attain an effective dual primer sets amplification in a single tube, annealing temperatures and primer concentrations were optimized. The annealing temperature for outer primers was designed to be higher than that of inner primers to allow separation of two rounds of PCR successfully. Figure 7 shows the conventional PCR with inner primers at temperatures ranging from 50°C to 65°C. No amplicons were found at temperatures above 62.6°C. Strong bands of expected size were observed at temperatures between 53°C and 57°C. The conventional PCR with outer primers was performed at temperatures ranging from 60°C to 70°C. No amplicons were found at temperatures over 69.7°C. Strong bands of expected size were observed at temperatures between 60°C and 67°C. Considering that the inner primers should not amplify any

products during the first round of PCR, optimum annealing temperatures for inner primers and outer primers were determined to be 55°C and 65°C, respectively. The ratio of outer primers and inner primers concentrations was optimized to ensure that no visible outer primer products would be observed. Figure 8 demonstrated that no visible outer primer products were retained with the outer primer concentrations of 0.05 and 0.1 pmol. Moreover, the increase in inner primer concentration resulted in a stronger band. Considering that higher concentration of outer primers could increase the detection sensitivity, the optimum concentrations were determined to be 0.1 pmol for outer primers and 40 pmol for inner primers.

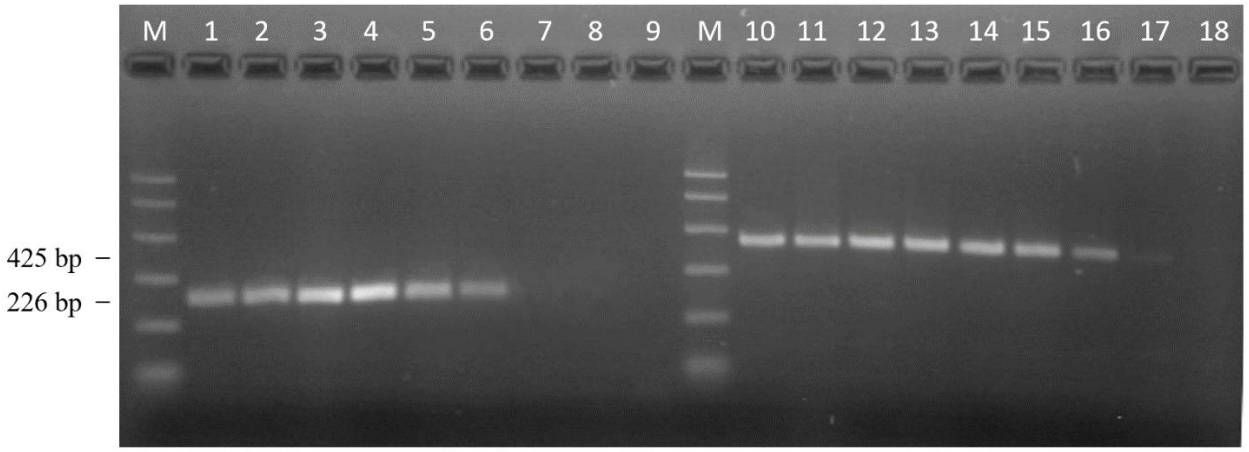

Figure 7: Optimization of the annealing temperatures for inner primers and outer primers. Lanes 1-8 represent the annealing temperature for inner primers at 50.0, 51.1, 53.2, 55.9, 59.3, 62.0, 64.0, and 65.0^{o}C. Lanes 10-17 represent the annealing temperature for outer primers at 60.0, 60.8, 62.1, 64.0, 66.2, 68.1, 69.4, and 70.0^{o}C. Lane 9 and lane 18 are water control. M is PCR markers.

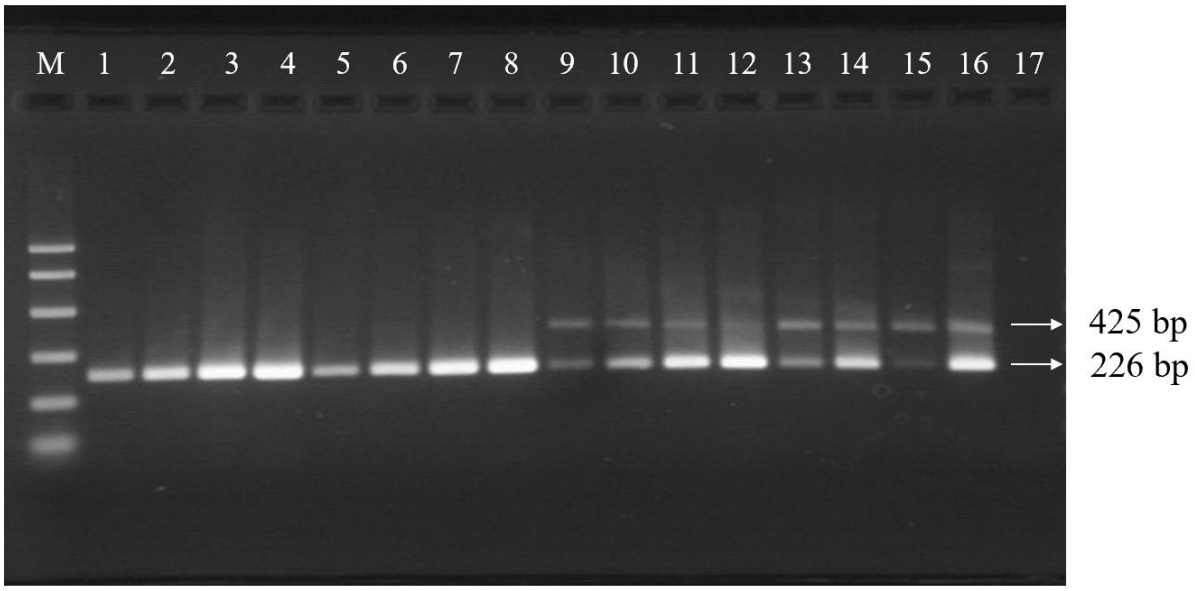

Figure 8: Optimization of ratios of outer and inner primer concentrations in STN-PCR. Lanes 1-16 represent 0.05:5, 0.05:10, 0.05:20, 0.05:40, 0.1:5, 0.1:10, 0.1:20, 0.1:40, 0.5:5, 0.5:10, 0.5:20, 0.5:40, 1:5, 1:10, 1:20, and 1:40 of outer and inner primer ratios. Lane 17 is water control. M is PCR markers.

3.3.3 Specificity test

The specificity of four designed primers was evaluated in silico against all microbial DNA sequences in NCBI databases. The BLAST searches showed these two pairs of primers were highly specific to *C. jejuni*. The specificity was further confirmed by the STN-PCR assay with DNA extracted from three *C. jejuni*, two *C. coli, C. lari*, and seven non-*Campylobacter* bacterial strains. Figure 9 showed the established STN-PCR assay only generated expected amplicons from *C. jejuni* DNA.

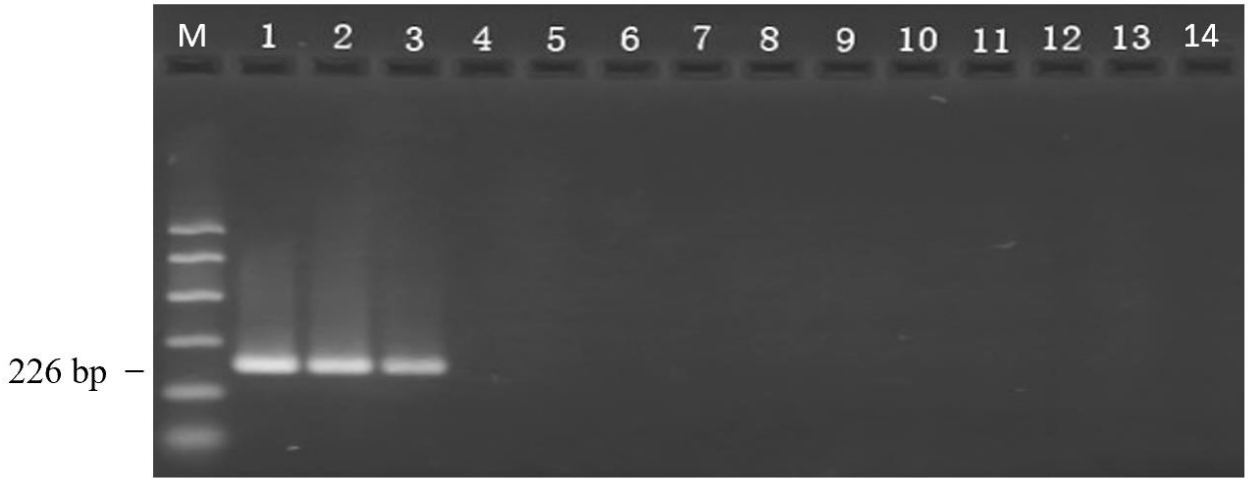

Figure 9: Specificity test of the established STN-PCR assay. Lanes 1-13 represent *C. jejuni* 11168, *C. jejuni* Penn 4, *C. jejuni* Penn 19, *C. coli* clinical, *C. coli* 5-, *C. lari*, *Proteus vulgaris*, *Bacillus cereus*, *Staphylococcus aureus*, *Shigella flexneri*, *Listeria monocytogenes*, *E. coli* O157: H7 and *Salmonella* Typhimurium. Lane 14 is water control, and M is PCR markers.

3.3.4 Comparison of conventional PCR and STN-PCR

Pure *C. jejuni* DNA was serially diluted in DNase-free water, representing 10^7 to single copies of *C. jejuni* DNA. The sensitivity of the STN-PCR assay was determined with the *C. jejuni* DNA copies as standards and compared with that of conventional PCR. Figure 10 showed that the detection limit of conventional PCR with inner primers was 10^3 copies of *C. jejuni* DNA. In comparison, the STN-PCR assay generated positive results with as few as 10 copies of *C. jejuni* DNA, which was 100 times more sensitive than conventional PCR.

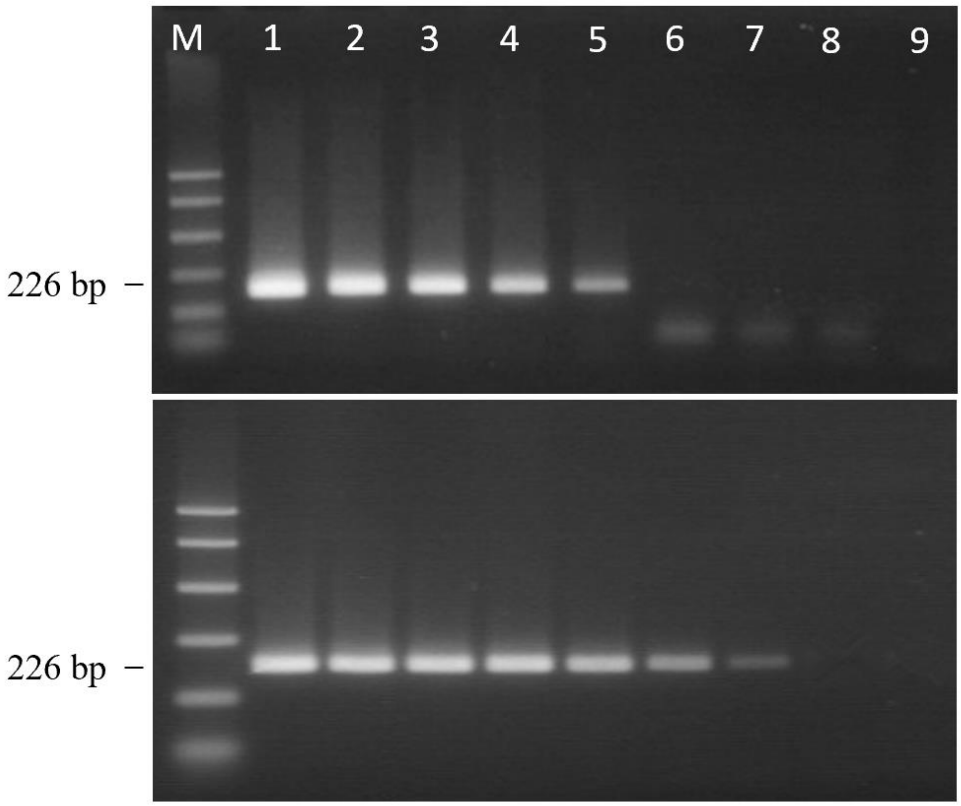

Figure 10: Conventional PCR with inner primers (top) and STN-PCR (bottom) with DNA extracted from *C. jejuni* culture. Lanes 1-8 represent 10^7, 10^6, 10^5, 10^4, 10^3, 10^2, 10, 5 copies of C. *jejuni* DNA. Lane 9 is water control, and M is PCR markers.

3.3.5 Detection of *C. jejuni* in artificially contaminated ground chicken homogenate

Total DNA extracted from artificially contaminated ground chicken homogenate was used to further evaluate the performance of the established assay. The sensitivity of STN-PCR with chicken samples was also compared with that of conventional PCR (Figure 11). The detection limit of conventional PCR was 3.6×10^3 CFU/ml *C. jejuni* in ground chicken homogenate, whereas the STN-PCR assay successfully detected *C. jejuni* at as low as 3.6×10^1 CFU/ml in ground chicken homogenate. Thus, the STN-PCR assay again showed sensitivity 100 times higher than that of conventional PCR in the presence of chicken constituents.

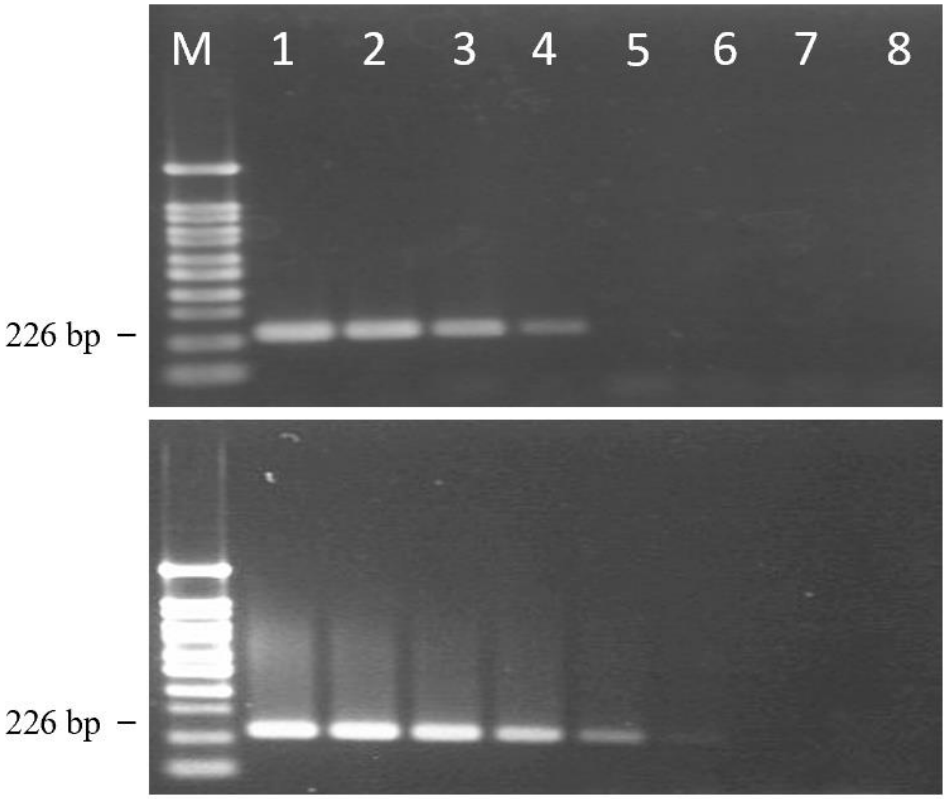

Figure 11: Conventional PCR with inner primers (top) and STN-PCR (bottom) with total DNA extracted from ground chicken homogenate. Lanes 1-6 represent 3.6×10^6 CFU/ml to 3.6×10^1 CFU/ml *C. jejuni* in ground chicken homogenate. Lane 7 is uninoculated ground chicken homogenate, and Lane 8 is water control. M is 1000-bp PCR marker.

3.3.6 Quantification of *C. jejuni* in ground chicken homogenate by STN-rtPCR

SYBR green real-time PCR was applied to quantify *C. jejuni* in ground chicken homogenate and compare the amplification efficiency of STN-rtPCR assay and conventional rtPCR with inner primers (Figure 12). Standard curves for both assays were constructed. All uninoculated control samples and water control tested negative. The detection limit of conventional rtPCR was 10^3 CFU/ml *C. jejuni* with Ct value of 28.10 ± 0.74. In comparison, the detection limit of STN-rtPCR was determined as 3.6×10^1 CFU/ml *C. jejuni* in samples with Ct value of 25.08 ± 0.10, which is much more sensitive than conventional rtPCR. The efficiency of conventional rtPCR was 100%, and that of single tube nested real-time PCR was 96.33%. The correlation coefficients of these two assays were 0.9951 and 0.9984.

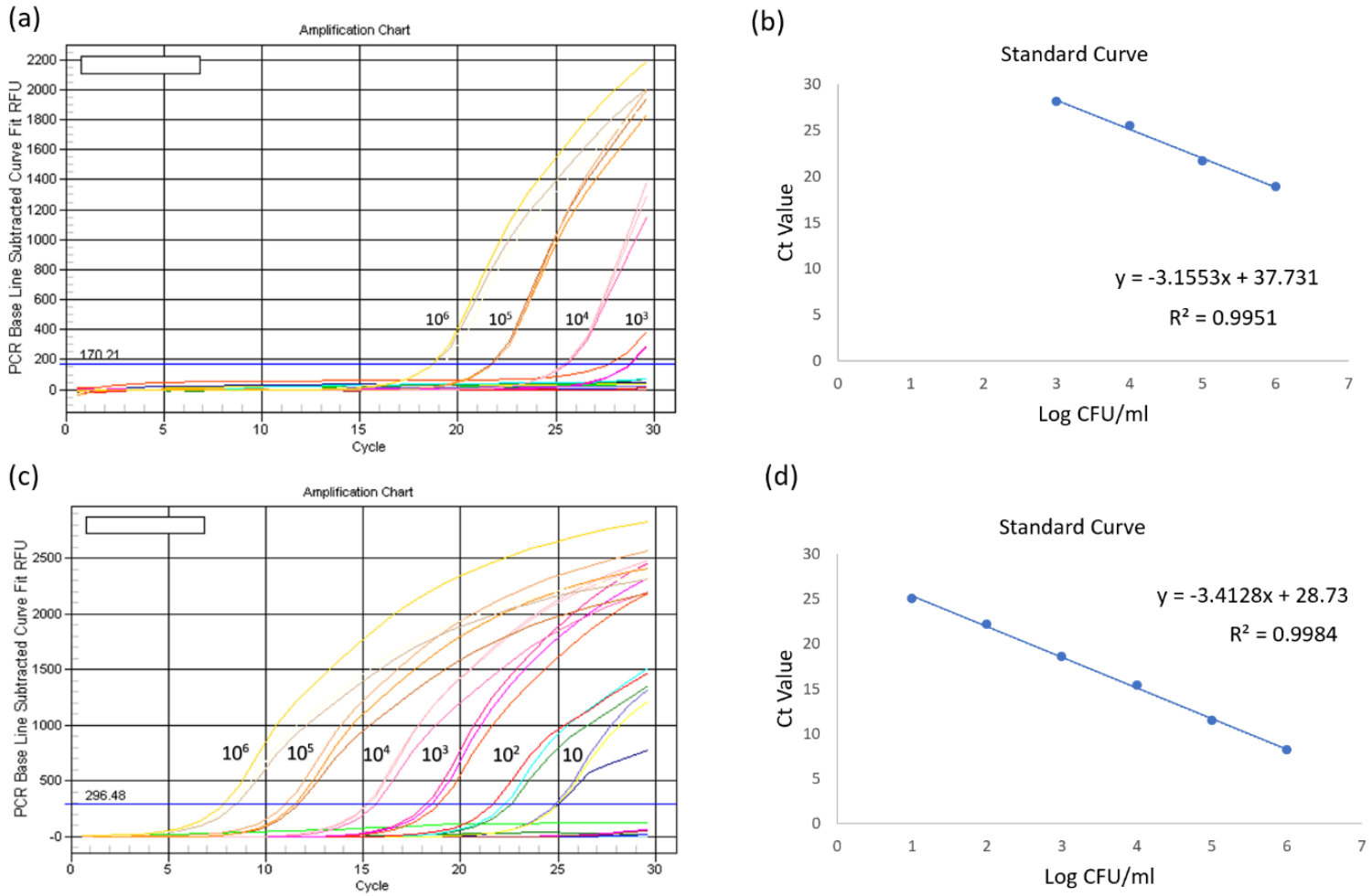

Figure 12: Comparison of the sensitivity of single tube nested real-time PCR with real-time PCR with inner primers in detecting *C. jejuni* ranging from 3.6×10^6 CFU/ml to 3.6×10 CFU/ml in artificially contaminated ground chicken homogenate. (a) Real-time amplification plot of real-time PCR; (b) standard curve generated by real-time PCR amplification; (c) Real-time amplification plot of single tube nested real-time PCR; (d) standard curve generated by single tube nested real-time PCR amplification.

3.3.7 Comparison of three methods for detecting levels of *C. jejuni* in artificially inoculated ground chicken homogenate with enrichment

Culture-based method, conventional rtPCR and STN-rtPCR were compared for their performance in detecting *C. jejuni* in artificially inoculated ground chicken homogenate (Table 1). It was determined that an initial inoculum of 0.1 CFU/g was detectable by STN-rtPCR after 24 h of enrichment, while it was tested positive by both culture-based method and conventional rtPCR after 48 h of enrichment. Without enrichment, the detection limits for STN-rtPCR and conventional rtPCR were 10^1 CFU/g and 10^3 CFU/g, respectively, while neither inoculation level was detectable by the culture-based method. The ground chicken homogenate containing 10^0 CFU/g *C. jejun* were identified by STN-rtPCR after 6 h of enrichment. In comparison, in order to detect inoculation levels of 10^2 CFU/g, 10^1 CFU/g, and 10^0 CFU/g by conventional rtPCR, enrichment times of 12 h, 24 h, and 48 h were required, respectively. Nevertheless, when the culture-based method was used, 10 h, 24 h, and 48 h of enrichment were necessary to identify *C. jejuni* of 10^3 CFU/g, 10^1 CFU/g, and 10^{-1} CFU/g, respectively, in ground chicken homogenate. The suspectable colonies picked from mCCDA that were confirmed to be non-*C. jejuni* strains were further sequenced and identified as *Enterococcus faecalis* and *Citrobacter freundii*. These two bacteria created colonies on mCCDA showing similar morphotype to *C. jejuni*. Therefore, the culture-based method posed a great challenge of generating false-positive results. These observations revealed that the established STN-rtPCR assay was significantly more efficient than the other two methods in analyzing ground chicken samples that contained low levels of *C. jenuni*.

Table 1: Comparison of culture-based method, conventional rtPCR, and STN-rtPCR in detecting low levels of *C. jejuni* in ground chicken homogenate with enrichment.[a]

Incubation time (h)	Detection method	Inoculation level (CFU/g)				
		10^{-1}	10^{0}	10^{1}	10^{2}	10^{3}
0	Culture-based	-[b]	-	-	-	-
	Conventional rtPCR[c]	-	-	-	-	28.11[d]±0.03[e]
	STN-rtPCR[f]	-	-	26.17±0.61	22.64±0.50	20.16±0.20
2	Culture-based	-	-	-	-	-
	Conventional rtPCR	-	-	-	-	28.90±0.16
	STN-rtPCR	-	-	25.63±0.72	21.88±0.14	20.68±0.15
4	Culture-based	-	-	-	-	-
	Conventional rtPCR	-	-	-	-	28.02±0.67
	STN-rtPCR	-	-	23.72±0.72	22.19±0.38	21.16±0.42
6	Culture-based	-	-	-	-	-
	Conventional rtPCR	-	-	-	-	27.89±0.56
	STN-rtPCR	-	26.79±0.88	22.61±0.21	20.66±0.16	19.32±0.68
8	Culture-based	-	-	0/3[g]	-	0/3
	Conventional rtPCR	-	-	-	-	27.87±0.58
	STN-rtPCR	-	25.11±0.74	22.32±0.33	20.00±0.48	19.20±0.34

Table 1 (continued).

10	Culture-based	-	-	0/3	0/3	1/3
	Conventional rtPCR	-	-	-	-	27.27±0.14
	STN-rtPCR	-	24.44±0.54	21.97±0.12	18.40±0.27	17.95±0.22
12	Culture-based	-	-	0/3	0/3	3/3
	Conventional rtPCR	-	-	-	27.41±0.14	25.55±0.39
	STN-rtPCR	-	23.38±0.64	21.33±0.53	16.81±0.36	15.66±0.16
24	Culture-based	0/3	0/3	2/3	3/3	3/3
	Conventional rtPCR	-	25.34±0.08	23.73±0.51	19.26±0.19	17.60±0.21
	STN-rtPCR	18.09±0.53	13.52±0.79	13.47±1.20	8.28±1.04	5.39±0.33
48	Culture-based	3/3	3/3	3/3	3/3	3/3
	Conventional rtPCR	10.73±0.60	10.67±0.73	12.12±0.59	10.05±0.06	11.26±0.51
	STN-rtPCR	3.71±0.49	1.00±0.01	1.73±1.04	1.00±0.01	1.00±0.01

[a] No amplicon was observed from uninoculated ground chicken homogenate.
[b] No fluorescence signal detected after 30 cycles was shown as -.
[c] Conventional real-time PCR with inner primers.
[d] Mean of Ct values is averaged from three replicates.
[e] Standard deviation of Ct values.
[f] Single-tube nested real-time PCR.
[g] Suspectable colonies confirmed *C. jejuni* positive or negative by single-tube nested real-time PCR.

3.4 Discussion

The ability of a microbiological method to rapidly detect pathogens in food products is critical because it can provide valuable information on occurrence of contaminants and assist manufacturers in producing safe food for the consumer. *Campylobacter* is the leading cause of bacterial diarrheal illness in the United States (Marder *et al*., 2018). The incidence of *C. jejuni* in patients is typically associated with acute diarrhea with abdominal pain and watery or bloody diarrhea lasting a few days. Generally, *Campylobacter* infections are caused by consuming contaminated food or contacting infected animals or pets. To protect the public health, rapid and reliable detection methods are essential.

The conventional cultural method is laborious and time-consuming. The FDA recommended bacteriological analytical manual for *Campylobacter* testing requires at least four days to enrich and isolate *Campylobacter* in food. Therefore, numerous molecular assays have been introduced for rapid detection of *C. jejuni* from a spectrum of food types. Nevertheless, most of these assays are not able to achieve rapid and highly sensitive detection of low levels of *C. jejuni* in food (Sabike and Yamazaki, 2019). Nested PCR is a composition of two rounds of PCR. In this way, the specificity is significantly improved by amplifying an internal region of the products from the first round of PCR. Several studies have applied this technique for the detection of *Campylobacter* species. Winters *et al*. (1997) introduced a nested PCR for identification of *C. jejuni* in pure culture at a sensitivity of 10^2 CFU/ml. Júnior *et al*. (2003) described a 16S rDNA-based nested PCR to detect *Campylobacter gracilis* in samples from root canal infections, abscesses, and subgingival plaque. The detection limit was determined to be 10 *C. gracilis* cells by amplifying serial dilutions of *C. gracilis* genomic DNA. Bang *et al*. (2002) developed a nested PCR assay to detect *C. jejuni* and *C. coli* in environmental samples from broiler farms.

They introduced two combinations of nested primer sets targeting on 16S rRNA gene of *C. coli* and *C. jejuni*, and *hip*O gene of *C. jejuni*. The sensitivity of the nested PCR assays was determined as low as 2-3 CFU/ml using *C. jejuni* DNA as templates.

However, since nested PCR includes two rounds of PCR amplification, it requires the first PCR amplification products to be transferred into another reaction tube for the second round of amplification, which can potentially cause DNA cross-contamination and false-positive results. Moreover, an additional round of PCR amplification makes this procedure time-consuming and doubles the cost of reagents (Lin *et al*., 2016). A novel method called single tube nested PCR (STN-PCR) has been developed to overcome these deficiencies, which is processed via combining both rounds of PCR amplification in a single closed tube. Well-designed primers/probe and optimization of the amplification protocol are essential for fulfilling the potential of STN-PCR (Lin *et al*., 2010). In our study, both primer sets were designed based on the hippuricase gene which can specifically discriminate *C. jejuni* from other *Campylobacter* species (Hani *et al*., 1995). Moreover, the formation of primer-dimers and hairpin structures was minimized. *In silico* verification demonstrated that the two primer sets used in this study could only amplify *C. jejuni* DNA. Optimal PCR conditions are also critical, as the differences of annealing temperature between outer primers and inner primers would allow successful separation of two rounds of PCR amplification within one tube. Furthermore, the appropriate ratio of inner and outer primers would yield expected amplification products with high quality. Our results indicated 0.1 pmol outer primers at 55 C for 20 cycles followed by 40 pmol inner primers at 65°C for 30 cycles are favorable for the detection of *C. jejuni* by STN-PCR.

The sensitivity of established STN-PCR assay was compared with conventional PCR only using inner primers. Previous studies showed that the conventional real-time PCR could detect

10^3 *C. jejuni* CFU/g broiler feces (De Boer *et al*., 2015; Lund *et al*., 2004). Lin *et al*. (2008) reported the concentrations of 10^2 CFU/ml and 10^3 CFU/ml *C. jejuni* in pure culture and human stool samples were detectable by real-time PCR, respectively. In addition, Papić *et al*. (2017) confirmed that 10^4 to 10^3 CFU/g of *C. jejuni* in chicken skin homogenate were the lowest concentration that their method could detect. In this study, the detection limit of STN-PCR was determined to be 10 copies of *C. jejuni* DNA, which was 100 times more sensitive than conventional PCR. Similar results were obtained from experiments with artificially contaminated ground chicken homogenate, in which STN-PCR and conventional PCR yielded detection limits of 36 CFU/ml and 3.6×10^3 CFU/ml, respectively. Further quantification of *C. jejuni* by STN-rtPCR provided a linear relationship between the threshold cycles and the log of *C. jejuni* cells in ground chicken homogenate with a correlation coefficient of 0.9984. The assay offers a promising tool for sensitive detection and quantification of *C. jejuni* in ground chicken.

A thorough comparison of culture-based method, conventional rtPCR and STN-rtPCR was conducted to detect 10^{-1} CFU/g to 10^3 CFU/g *C. jejuni* in artificially contaminated ground chicken homogenate at different enrichment times. In order to detect the lowest inoculation level of 10^{-1} CFU/g, the STN-rtPCR assay required 24 h of enrichment. However, 48 h of enrichment, which is generally recommended by FDA and USDA, was needed for culture-based method and conventional rtPCR to detect the same inoculation level. Giesendorf *et al*. (1992) inoculated 25 CFU/g of *C. jejuni* in chicken meat samples and incubated them for 12, 14, 16, and 18 hours. The DNA from the samples at different enrichment times was extracted and applied to real-time PCR. They concluded that a minimal enrichment time of 18 h was required to detect 25 CFU *C. jejuni* per gram chicken meat. In the present study, a similar result was observed that an inoculation level of 10^1 CFU/g in the ground chicken homogenate was only detectable by

conventional rtPCR after 24 h of enrichment. Moreover, enrichment of 24 h was sufficient to identify a concentration of 10^0 CFU/g *C. jejuni* in ground chicken meat by conventional rtPCR. Therefore, a shorter enrichment time of 24 h was widely applied in PCR assays for the detection of *C. jejuni*, which could also avoid the overgrowth of competing bacteria (Chon *et al*., 2011; Odongo *et al*., 2009). Overall, the developed STN-PCR assay presented superior performance in detecting very low levels (10^{-1} CFU/g and 10^0 CFU/g) of *C. jejuni* in ground chicken, which would allow food manufacturers to effectively monitor their products and make sound and timely decisions to protect the public health.

The culture-based assay conducted in this study displayed a potential for yielding false positive results. The suspected colonies picked from mCCDA that had been confirmed as non-*C. jejuni* strains were further identified to be *Enterococcus faecalis* and *Citrobacter freundii* by DNA sequencing. A study conducted by Chon *et al*. (2012) determined that, except for *C. jejuni* and *C. coli*, eight non-*Campylobacter* strains were also able to grow on mCCDA, including *Enterococcus* spp. and *Citrobacter freundii*. In their report, the performance of Preston agar, mCCDA, and Campy-Cefex agar were compared for the detection of *Campylobacter* in chicken carcass rinse samples, among which the Preston agar demonstrated a significantly better isolation rate of *Campylobacter* and less growth by competing microflora. Nevertheless, the Campy-Cefex agar that is recommended by USDA Food Safety and Inspection Service to isolate *Campylobacter* spp. from poultry samples (USDA-FSIS, 2016) exhibited the lowest isolation rate and highest contamination by competing bacteria. Though, sample types should be taken into consideration. It was reported that mCCDA performed better than Preston agar with human feces sample (Corry *et al*., 1995). However, Peterz (1991) presented that the Preston agar and mCCDA showed similar sensitivity and selectivity with fewer contaminants on mCCDA than on

Preston agar for the detection of *C. jejuni* in chicken liver. Chon *et al*. (2011) confirmed that Preston agar was more selective than mCCDA using ground beef and fresh-cut vegetables. On the other hand, the supplements added to both enrichment broth and isolation agar could affect the specificity and sensitivity of detection for *C. jejuni*, and the efficiency of inhibition against diverse background bacteria (Chon *et al*., 2013a; Chon *et al*., 2013b; Chon *et al*., 2014a; Chon *et al*., 2014b; Chon *et al*., 2017; Jo *et al*., 2017; Seliwiorstow *et al*., 2016). Nevertheless, the validation of highly selective and efficient culture-based assays remains necessary to distinguish the etiological agents of clinical diseases (Kim *et al*., 2016).

To our knowledge, the present study is the first report demonstrating STN-PCR for the detection of foodborne pathogenic bacteria. Our results indicate that the established STN-PCR assay offers an effective tool for rapid detection of *C. jejuni* in ground chicken. This assay exhibits the ability to eliminate the potential of target DNA cross-contamination that is associated with nested PCR and has very low detection limit that is superior to conventional PCR. The next step is to investigate its applicability to naturally contaminated ground chicken from local grocery stores and broader sample types. In particular, the sample preparation method can be adjusted toward complex food matrix. Furthermore, the enrichment step may be optimized to improve the performance of established STN-PCR assay.

Chapter 4

Development of a Multiplex Single-tube Nested Real-time PCR Assay for Simultaneous Detection of *Campylobacter jejuni* and *Salmonella* spp.

4.1 Introduction

Campylobacter jejuni and *Salmonella* spp. are the most common foodborne pathogenic bacteria which cause human gastroenteritis in the United States and worldwide (Alves *et al*., 2016). With the use of culture-independent diagnostic tests (CIDTs), the incidences of *Campylobacter* and *Salmonella* infections in 2018 were determined to be 9,723 and 9,084, respectively (Tack *et al*., 2019). Moreover, CDC estimates that more than 1.3 million individuals get infected by *Campylobacter* strains per year in the United States, while most cases are undiagnosed or unreported (Marder *et al*., 2018). It is also estimated that *Salmonella* infections due to ingestion of contaminated food account for 1 million illnesses, 19,000 hospitalizations, and 380 deaths yearly (CDC, 2019). The economic loss of foodborne illnesses caused by *Campylobacter* and *Salmonella* is $1.9 billion and $5.4 billion, respectively, including medical cost, productively loss, and premature death (Scharff, 2015; Scharff, 2018).

Poultry is considered to be a primary source of *Campylobacter*- and *Salmonella*- associated human diseases, which cost approximately $2.0 billion annually (Hill *et al*., 2017). Out of the foodborne diseases attributed to poultry, *Campylobacter* accounts for 58% followed by *Salmonella* at 28% (Hill *et al*., 2017; Morris *et al*., 2011; Nagel *et al*., 2013). In 2018, a new enrichment method was used by the USDA Food Safety and Inspection Service (USDA-FSIS) to detect *Salmonella* and *Campylobacter* in raw poultry samples.18% of chicken carcasses and 16% of chicken parts were tested positive for *Campylobacter*. In addition, chicken parts collected

from 22% of food processing facilities yielded *Salmonella* (Tack *et al*., 2019).

The standard methods employed to determine the presence of pathogenic bacteria in food involve enriching food samples with nutritious broth, isolating targets on selective agar, and identifying typical colonies according to their morphological, biochemical, and/or immunological characteristics, which may take 5-6 days to complete (Li and Mustapha, 2004). Currently, molecular methodsbecome prevailing due to their powerful ability to allow for sensitive and specific detection (Gasanov *et al*., 2005), of which the polymerase chain reaction (PCR) is considered to be a promising technique for accurate identification of target organisms. Besides, two-step nested PCR has been used for highly sensitive detection of pathogenic bacteria including *Salmonella* and *C. jejuni* (Bang *et al*., 2002; Liu *et al*., 2002; Rychlik *et al*., 1999; Saroj *et al*., 2008; Waage *et al*., 1999; Winters *et al*., 1997). However, nested PCR involves the transfer of amplified products from first-round PCR to another tube for a second-round PCR, which increases the potential for cross-contamination due to additional manipulation of amplicons (Dey *et al*., 2012; Hamim *et al*., 2018; Lin *et al*., 2010). A solution to this problem is to integrate the two-tube nested PCR into a single-tube reaction (Dey *et al.,* 2012; Kemp *et al*., 1990; Orou *et al*., 1995). The single-tube nested PCR (STN-PCR) has been successfully applied to detect human viruses, plant viruses, parasites, fungi, and bacterial toxins (Burkhardt *et al*., 2018; Dey *et al*., 2012; Feng *et al*., 2018; Hamim *et al*., 2018; Jang *et al*., 2015; Klemsdal and Elen, 2006; Llop *et al*., 2000; Saito *et al*., 2018).

Notably, there is a chance that *Salmonella* and *C. jejuni* might contaminate similar types of food, such as poultry products. Therefore, it is desirable to detect multiple pathogens in the same reaction as it would significantly reduce the cost and time for food testing. This study aimed to develop a multiplex single-tube nested real-time PCR (STN-rtPCR) assay for simultaneous

detection of *Salmonella* and *C. jejuni*. The performance of the developed assay was evaluated with artificially contaminated chicken rinse.

4.2 Materials and Methods

4.2.1 Bacterial strains and culture conditions

The bacterial strains used in this study included *C. jejuni* ATCC 11168, *C. jejuni* Penn 4, *C. jejuni* Penn 19, *Salmonella* Typhimurium UMC, *Salmonella* Typhimurium ATCC 14028, *Salmonella* Enteritidis, *C. coli* clinical, *C. coli* Penn 5, *C. lari, Proteus vulgaris*, *Bacillus cereus*, *Staphylococcus aureus*, *Shigella flexneri*, *Listeria monocytogenes*, and *Escherichia coli* O157: H7. These strains were obtained from Food Microbiology Lab collections at the University of Hawaii at Manoa. All strains were stored in 50 % glycerol at -80 °C before use. All *Campylobacter* strains were cultivated in Muller Hinton broth (Becton Dickinson) at 42°C for 48 h in microaerophilic GasPak™ EZ Campy pouches. *Salmonella* and other bacterial strains were aerobically grown in trypticase soy broth (Becton Dickinson) at 37°C for 24 h.

4.2.2 DNA extraction

The DNeasy Blood & Tissue Kit (QIAGEN, Germany) and PrepMan Ultra reagent (Applied Biosystems, UK) were used for DNA extraction. Genomic DNA of *C. jejuni* ATCC 11168 and *Salmonella* Typhimurium ATCC 14028 was extracted using a DNeasy Blood & Tissue Kit according to the instructions of the manufacturer for Gram-negative bacteria. The final DNA concentration was measured using the NanoDrop2000 spectrophotometer (Thermo Scientific, Waltham, USA). The purified DNA was serially diluted with DNase-free water to a final concentration of 100 ng/μL and stored at -20°C until use.

DNA of other thirteen bacterial cultures was extracted by PrepMan Ultra reagent. Briefly, 0.1

ml of bacterial culture was transferred to a 1.5 ml microcentrifuge tube and centrifuge at 13,200 rpm for 1 min. Cell pellets obtained were resuspended in 0.1 ml PrepMan Ultra reagent and placed in boiling water for 10 min. After cooling down, the mixture was centrifuged at 13,200 rpm for 2 min. The supernatant was collected and used as template DNA in PCR assay.

4.2.3 Design of primers and fluorogenic probes

Two sets of primers based on the hippuricase (*hip*O*)* gene of *C. jejuni* and two sets of primers based on the *inv*A gene of *Salmonella* were designed using the Primer3 software (Table 2). The criteria for inner primers were shown as follows: GC% ≥ 40-50, Tm = 55± 2°C, primer length = 16-22 bp, and amplicon size of 100-300 bp. The same criteria were also applied to the design of outer primers, except that a higher annealing temperature (at least 10°C higher than inner primers) and longer amplicon size (300-500 bp) were considered. The AmplifX software (http://crn2m.univ-mrs.fr/pub/amplifx-dist) was used to locate primers within the target gene sequences and analyze the quality of designed primers to select the best inner and outer primers sets with the least possibility of forming self/cross dimers. Primers were also validated *in silico* against all microbial DNA sequences in NCBI databases (https://www.ncbi.nlm.nih.gov/).

Specific TaqMan® probes for *C. jejuni* and *Salmonella* were designed using PrimerQuest Tool (Integrated DNA Technologies, USA). The 5'-end of probes for *C. jejuni* and *Salmonella* were labeled with FAM and TAMRA fluorophores, respectively. The 3'-end of probes for *C. jejuni* and *Salmonella* were labeled with BHQ-1 and BHQ-2, respectively (Table 2). All probes were customized by Sigma-Aldrich (USA). The annealing temperature of probes was designed to be 10°C higher than that of inner primers.

Table 2. Primers and probes used in the multiplex single-tube nested real-time PCR assay for the detection of *C. jejuni* and *Salmonella* spp.

Bacteria	Target gene	Primer/probe	Sequence (5'-3')	Amplicon size (bp)
Campylobacter jejuni	*hip*O	C-outer-F	AACAGGCGTTGTGGGGGTTT	425
		C-outer-R	TTCCATGACCACCCCTTCCA	
		C-inner-F	ATAGGACTTCGTGCAGATA	226
		C-inner-R	CTTCTATCATTGCCTTAGC	
		C-probe	[6-FAM] ATTTTCAACCTGCTGAAGAGGGTTT [BHQ-1][a]	
Salmonella spp.	*inv*A	S-outer-F	GTCGCCCAGATCCCCGCATT	293
		S-outer-R	CTGAGCGGCTGCTCGCCTTT	
		S-inner-F	TTGAACAACCCATTTGTATT	173
		S-inner-R	CCTTTGCTGGTTTTAGGTTT	
		S-probe	[TAMRA] AACTCTGCCGGGATTCCCACT [BHQ-2][a]	

[a] TAMRA (carboxytetramethylrhodamine); BHQ-1 (Black-hole quencher 1); 6-FAM™ (6-carboxyfluorescein); BHQ-2 (Black-hole quencher)

4.2.4 Development of multiplex single-tube nested PCR

First, the efficiency of uniplex single-tube nested PCR (STN-PCR) assay was evaluated by using ten-fold serial dilutions of pure DNA extracted from *S.* Typhimurium or *C. jejuni* cultures, ranging from 10 ng to 10 fg per reaction. The uniplex STN-PCR conditions for the detection of *Salmonella* spp. were as follows, 4 min at 95°C, followed by 20 cycles of 45 s at 95°C, 45 s at 65°C, extension at 72 °C for 45 s, and 30 cycles of 45 s at 95°C, 45 s at 55°C, extension at 72°C for 45 s, and a final extension at 72°C for 5 min. A reaction volume of 25 µl of uniplex STN-PCR mixture contained 1 µl DNA of *C. jejuni* or *S.* Typhimurium, 1 µl of 0.1 pmol outer primers, 1 µl of 40 pmol inner primers, 10.5 µl of Nuclease-free water, and 12.5 µl GoTaq ® Hot Start Colorless Master Mix (Promega, USA). The negative control was included by replacing the DNA with 1 µl of Nuclease-free water. The uniplex STN-PCR assay was conducted in a 48-well thermal cycler (MJ MINI PCR Thermal Cycler, Bio-Rad Laboratories, USA). Following the amplification, PCR products were analyzed by electrophoresis in 1.8% of agarose gels in 1× TAE buffer. The gels were visualized by UV light after being stained with GelGreen Nucleic Acid Stain (Biotium).

Subsequently, a multiplex single-tube nested PCR (STN-PCR) assay was developed based on the uniplex single-tube nested PCR assays for *C. jejuni* or *Salmonella* spp. A serial dilution of pure DNA from *S.* Typhimurium and *C. jejuni* were tested to optimize the concentration ratios of primers. The amounts of primers were set as 0.05, 0.1, 0.5 or 1.0 pmol for the outer primers, and 5, 10, 20 or 40 pmol for inner primers in a volume of 25 µl reaction mixture. The amplification conditions were set the same as uniplex STN-PCR. The reactions were carried out in duplicate, with a total volume of 25 µl, containing 1 µl DNA of *C. jejuni* and *S.* Typhimurium, 2 µl of *Salmonella* spp. primers, 2 µl of *C. jejuni* primers, 6.5 µl Nuclease-free water, and 12.5 µl

GoTaq ® Hot Start Colorless Master Mix. The negative control was included by replacing the DNA with 2 µl of Nuclease-free water. Following the amplification, PCR products were analyzed as described above.

4.2.5 Specificity of primers.

The genomic DNA extracts of 15 bacterial strains, including three *C. jejuni* strains, three *Salmonella* strains, two *C. coli* strains, *C. lari*, *Proteus vulgaris*, *Bacillus cereus*, *Staphylococcus aureus*, *Shigella flexneri*, *Listeria monocytogenes*, and *E. coli* O157: H7, were used as templates in the developed multiplex STN-PCR assay to evaluate the specificity of the designed primers.

4.2.6 Development of multiplex single-tube nested real-time PCR

The multiplex single-tube nested real-time PCR (STN-rtPCR) assay was conducted in the IQ5 System (Bio-Rad, CA, USA). The concentrations of probes were evaluated in a range of 1, 5, 10, and 20 pmol per reaction. All reactions contained 12.5 µl of TaqMan^{TM} Fast Advanced Master Mix (Applied Biosystems, USA), 1 µl of *S.* Typhimurium DNA, 1 µl of *C. jejuni* DNA, 2 µl of *Salmonella* primers, 2 µl of *C. jejuni* primers, 2 µl of each probe, and RNase free water to a final volume of 25 µl. A negative control was included by replacing the DNA with 2 µl of Nuclease-free water. The efficiency of uniplex and multiplex single-tube nested real-time PCR was evaluated with serial dilutions of pure DNA extracted from *S.* Typhimurium or *C. jejuni* culture, ranging from 10 ng to 10 fg per reaction.

The amplification conditions were 4 min at 95°C, followed by 20 cycles of 45 s at 95°C, 45 s at 65°C, extension at 72°C for 45 s, and 30 cycles of 45 s at 95°C, 45 s at 55°C, extension at 72°C for 45 s, and a final extension at 72°C for 5 min. Fluorescence data were collected at the end of each extension step of the inner primer pairs, and each sample was run in triplicate wells.

The Ct values (the cycle number at which fluorescence signal exceeds the fluorescence threshold), Ct mean and standard deviation of triplicates were calculated for each dilution. A standard curve with equation and R-squared value was generated for each bacterium by plotting the Ct mean from each dilution versus DNA concentration (Alves *et al*., 2016).

4.2.7 Evaluation of the developed multiplex single-tube nested real-time PCR assay with artificially contaminated chicken rinse

Artificially contaminated chicken rinse was prepared according to the method described by Alves *et al*. (2016) with minor modifications. Briefly, freshly packed boneless chicken was purchased from a local supermarket. Twenty-five grams of chicken were placed into a stomacher bag to which 225 ml of 0.1 % peptone water was added. The mixture was massaged manually for 1 min. The chicken rinse was aliquoted aseptically at a volume of 9 ml to sterile tubes (Falcon). One milliliter of *C. jejuni* or *Salmonella* pure culture was serially diluted with the chicken rinse to obtain approximately 10^0 CFU/ml to 10^7 CFU/ml. The inoculum concentration was confirmed by plate counting on Plate Count Agar (PCA, Becton Dickinson, USA) for *Salmonella* at 37ºC for 24 h and on Muller Hinton agar (Becton Dickinson, USA) for *C. jejuni* at 42ºC for 48 h in microaerophilic GasPak™ EZ Campy pouches. An uninoculated control was prepared by thoroughly mixing 9 ml chicken rinse with 1 ml of 0.1 % peptone water. In addition, the control was serially diluted in peptone water and plated on PCA followed by incubating at 37 ºC for 24 h to enumerate background microflora. After inoculation, total DNA of each chicken rinse sample was extracted using DNeasy Blood & Tissue Kit (QIAGEN, Germany), according to the instructions of the manufacturer for Gram-negative bacteria, and subjected to the established uniplex and multiplex single tube nested real-time PCR. This experiment was replicated three separate times.

4.3 Results

4.3.1 Development of multiplex single tube nested PCR

After a deliberate comparison of various reaction conditions was conducted, the optimal primer concentrations for the multiplex single-tube nested PCR (STN-PCR) were identified as 0.1 pmol *Salmonella* outer primers, 40 pmol *Salmonella* inner primers, 0.1 pmol *C. jejuni* outer primers, 20 pmol *C. jejuni* inner primers. The multiplex reaction yielded a 226 bp PCR product from *S.* Typhimurium and a 173 bp PCR product from *C. jejuni* (Figure 13).

The detection limit of the developed assay was determined in triplicated experiments using serially diluted DNA of *S.* Typhimurium and *C. jejuni* as templates. The concentrations of DNA ranged from 1×10^8 fg/ µl to 1 fg/µl. With visually suitable PCR fragments showed by agarose gel electrophoresis, the multiplex single tube nested PCR assay could reach a detection limit as low as 1 pg/µl of *Salmonella* and *C. jejuni* DNA simultaneously. Furthermore, the bands of expected sizes were comparable in intensity (Figure 13).

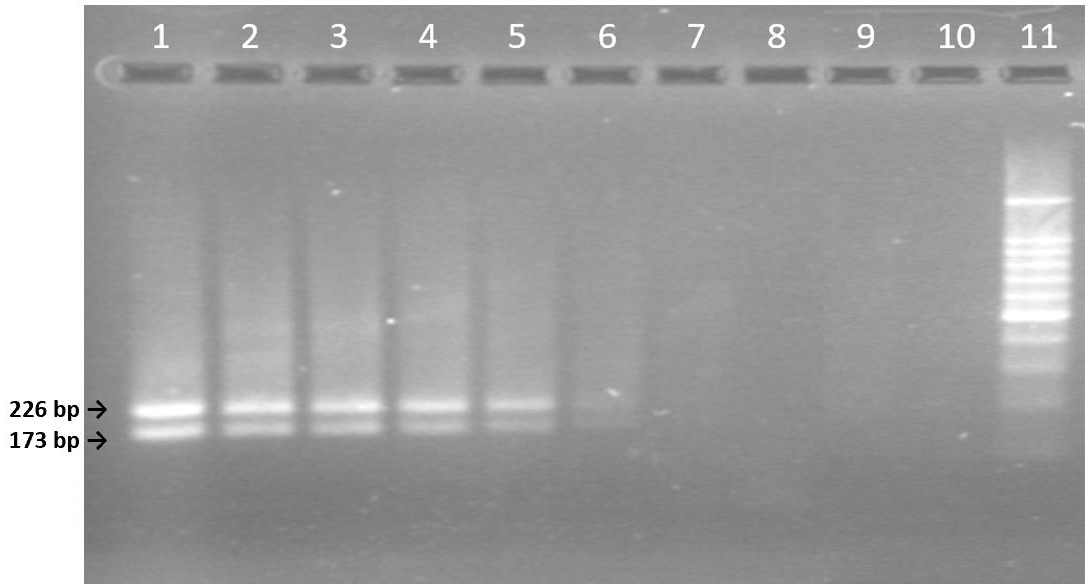

Figure 13: Agarose gel electrophoresis of amplicons from the optimized multiplex single tube nested PCR with pure DNA of *S.* Typhimurium and *C. jejuni*. Lanes 1-9 represent 1×10^{2} ng, 1×10^{1} ng, 1×10^{0} ng, 1×10^{-1} ng, 1×10^{-2} ng, 1×10^{-3} ng, 1×10^{-4} ng, 1×10^{-5} ng, and 1×10^{-6} ng of *S.* Typhimurium and *C. jejuni* DNA. Lane 10 is water control, and Lane 11 is 1000 bp PCR marker.

4.3.2 Specificity of the multiplex single tube nested PCR assay

The specificity of eight designed primers was investigated *in silico* against all microbial DNA sequences in NCBI databases. The BLAST searches identified these primers were highly specific to *Salmonella* spp. and *C. jejuni*. The specificity was further confirmed by multiplex STN-PCR with DNA extracted from three *C. jejuni*, two *C. coli*, *C. lari*, three *Salmonella* strains, and six other non-*Campylobacter* and non-*Salmonella* bacterial strains. Figure 14 showed that the established STN-PCR assay only generated expected amplicons from *Salmonella* spp. and *C. jejuni* DNA. No amplicons were observed when non-target DNAs were used as templates.

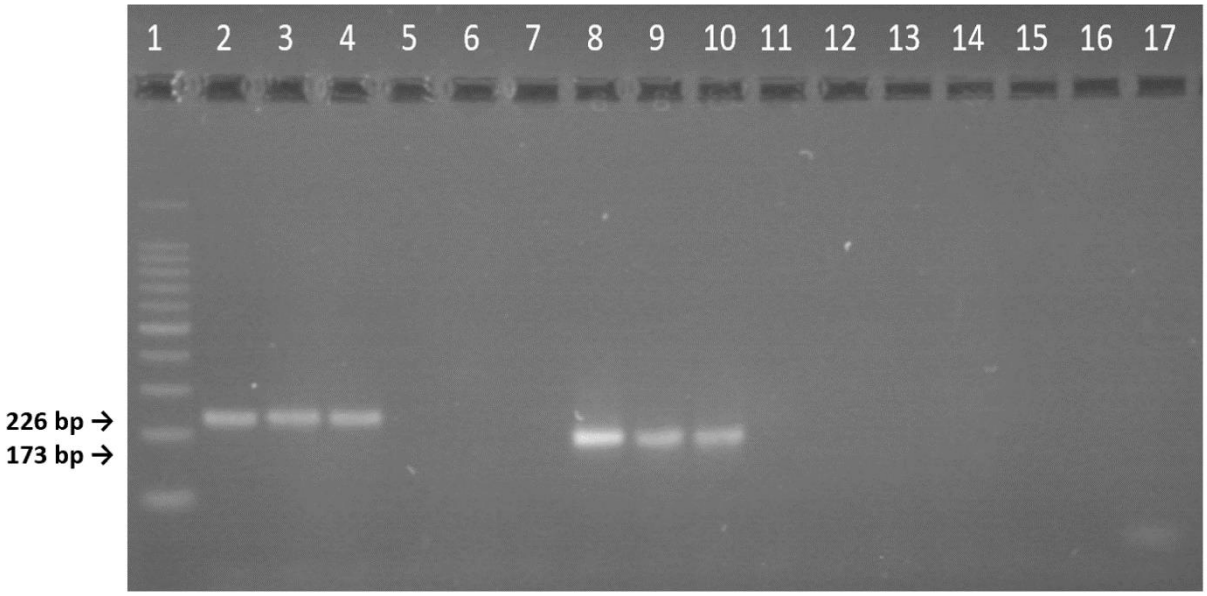

Figure 14: Specificity test of the established multiplex single tube nested PCR with DNA of different bacterial strains. Lanes 2-16 represent *C. jejuni* 11168, *C. jejuni* Penn 4, *C. jejuni* Penn 19, *C. coli* clinical, *C. coli* Penn 5-, *C. lari*, *Salmonella* Typhimurium UMC, *Salmonella* Typhimurium ATCC 14028, *Salmonella* Enteritidis, *Proteus vulgaris*, *Bacillus cereus*, *Staphylococcus aureus*, *Shigella flexneri*, *Listeria monocytogenes*, and *E. coli* O157: H7. Lane 1 is 1000 bp PCR marker, and lane 17 is water control.

4.3.3 Detection of *S.* Typhimurium and *C. jejuni* by the multiplex single tube nested real-time PCR

The developed multiplex single tube nested real-time PCR (STN-rtPCR) was evaluated for its ability to quantify DNA of *S.* Typhimurium and *C. jejuni* (Tables 3 and 4). Figure 15 depicted the standard curves obtained by plotting the Ct means from real-time PCR versus DNA concentrations of *S.* Typhimurium or *C. jejuni*. The data indicated that the detection limits for both pathogens were 10^{-3} ng/µl with R^2 values being 0.9988 and 0.999 for *S.* Typhimurium and *C. jejuni*, respectively. Besides, when equal amounts of *S.* Typhimurium DNA and *C. jejuni* DNA were applied to the multiplex STN-rtPCR at the same time, the sensitivity of this assay was not restrained. Besides, the Ct values for both target pathogens obtained from the multiplex STN-rtPCR (Table 4) were comparable to those from uniplex STN-rtPCR (Table 3). The differences in Ct values between uniplex STN-rtPCR and multiplex STN-rtPCR might be due to the competition of two amplification systems for limited reagents in the same reaction.

Table 3. Comparison of Ct values obtained by uniplex single tube nested real-time PCR assays with pure DNA of *S.* Typhimurium or *C. jejuni*.

DNA concentration (ng)	Ct value	
S. Typhimurium or *C. jejuni*	*S.* Typhimurium	*C. jejuni*
10^{1}	10.81[a] ± 0.45[b]	10.19 ± 0.66
10^{0}	14.93 ± 0.42	14.90 ± 0.16
10^{-1}	19.66 ± 0.30	19.69 ± 0.07
10^{-2}	23.90 ± 0.00	24.60 ± 0.57
10^{-3}	27.70 ± 0.52	28.63 ± 0.38

[a] Ct values are averages from three separate experiments.
[b] Standard deviation.

Table 4. Comparison of Ct values obtained by multiplex single tube nested real-time PCR assay with pure DNA of *S.* Typhimurium and *C. jejuni*.

DNA concentration (ng)		Ct value	
S. Typhimurium	*C. jejuni*	*S.* Typhimurium	*C. jejuni*
10^{1}	10^{1}	9.09[a] ± 0.65[b]	11.38 ± 0.86
10^{0}	10^{0}	13.88 ± 0.57	16.41 ± 0.46
10^{-1}	10^{-1}	19.04 ± 0.34	21.07 ± 0.27
10^{-2}	10^{-2}	23.71 ± 0.05	26.00 ± 0.20
10^{-3}	10^{-3}	28.72 ± 0.35	29.33 ± 0.24

[a] Ct values are averages from three separate experiments.
[b] Standard deviation.

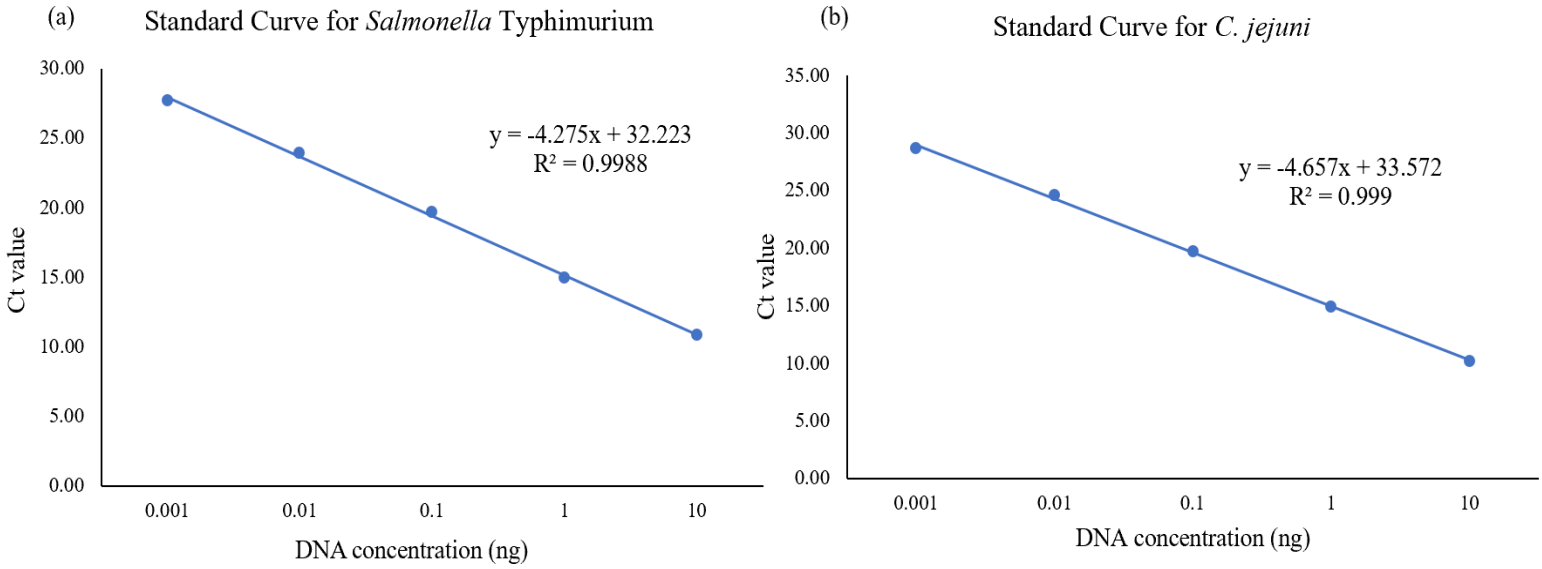

Figure 15: Standard curves generated by the uniplex single tube nested real-time PCR assays for quantifying *Salmonella* Typhimurium DNA (a) or *Campylobacter jejuni* DNA (b). The X-axis represents the concentration of pure DNA (ng/ul) from bacterial culture, and the Y-axis represents the cycle threshold (Ct) value.

Moreover, the effect of one pathogen on the detection of the other pathogen by the multiplex STN-rtPCR assay was evaluated. The Ct values for 10^{-1} ng/µl *S.* Typhimurium DNA remained stable around 19 in the presence of high and low concentrations of *C. jejuni* DNA (10^{1} ng/µl to 10^{-3} ng/µl) (Table 5). However, when the DNA concentration of *S.* Typhimurium was 10-fold or 100-fold higher than that of *C. jejuni*, the detection of *C. jejuni* in the same reaction by multiplex STN-rtPCR was completely inhibited (Table 6).

Table 5. Effect of *C. jejuni* on the detection of *S.* Typhimurium by developed multiplex single tube nested real-time PCR assay.

DNA concentration (ng/µl)		Ct value	
S. Typhimurium	*C. jejuni*	*S.* Typhimurium	*C. jejuni*
10^{-1}	10^{1}	19.94[a] ± 0.64[b]	11.85 ± 0.69
10^{-1}	10^{0}	18.70 ± 0.22	16.43 ± 0.44
10^{-1}	10^{-1}	19.09 ± 0.26	20.73 ± 0.13
10^{-1}	10^{-2}	18.92 ± 0.03	-[c]
10^{-1}	10^{-3}	18.99 ± 0.15	-

[a] Ct values are averages from three separate experiments.
[b] Standard deviation.
[c] No fluorescence signal detected after 30 cycles was shown as -.

Table 6. Effect of *S.* Typhimurium on the detectin of *C. jejuni* by developed multiplex single tube nested real-time PCR assay.

DNA concentration (ng/µl)		Ct value	
S. Typhimurium	*C. jejuni*	*S.* Typhimurium	*C. jejuni*
10^{1}	10^{-1}	9.72[a] ± 0.23[b]	-[c]
10^{0}	10^{-1}	14.67 ± 0.13	-
10^{-1}	10^{-1}	19.07 ± 0.44	21.06 ± 0.30
10^{-2}	10^{-1}	24.25 ± 0.53	21.07 ± 0.25
10^{-3}	10^{-1}	28.36 ± 0.31	21.60 ± 0.48

[a] Ct values are averages from three separate experiments.
[b] Standard deviation.
[c] No fluorescence signal detected after 30 cycles was shown as -.

4.3.4 Efficacy of the multiplex single tube nested real-time PCR assay in detecting *S.* Typhimurium and *C. jejuni* in artificially contaminated chicken rinse

The applicability of established multiplex STN-rtPCR assay was first evaluated with chicken rinse inoculated with *S.* Typhimurium or *C. jejuni*. All uninoculated control and water control tested negative. The detection limit of the uniplex STN-rtPCR assay was 10^2 CFU/ml for either pathogen in chicken rinse without enrichment. Differences in the Ct values for the same concentrations suggest that the amplification of *S.* Typhimurium DNA would be favored over that of *C. jejuni* DNA in the same reaction (Table 7).

Table 7. Evaluation of the developed multiplex single tube nested real-time PCR assay with chicken rinse inoculated with *S.* Typhimurium or *C. jejuni*. [a]

Bacterial concentration (CFU/ml)	Ct value	
S. Typhimurium or *C. jejuni*	*S.* Typhimurium	*C. jejuni*
10^7	5.07[b] ± 0.03[c]	11.04 ± 0.06
10^6	8.71 ± 0.00	13.61 ± 0.17
10^5	11.42 ± 0.17	16.34 ± 0.18
10^4	17.08 ± 0.16	19.80 ± 0.03
10^3	21.97 ± 0.13	23.33 ± 0.27
10^2	26.35 ± 0.17	27.91 ± 0.06
10^1	-[d]	-

[a] No amplicon was observed from uninoculated chicken meat rinse samples.
[b] Ct values are averages from three separate experiments.
[c] Standard deviation.
[d] No fluorescence signal detected after 30 cycles was shown as -.

The established multiplex STN-rtPCR assay was further evaluated with chicken rinse inoculated with both target pathogens. *S.* Typhimurium at concentrations of 10^2 CFU/ml to 10^6 CFU/ml were detected independent of tested *C. jejuni* concentrations, except that there was no amplification of *S.* Typhimurium at10^2 CFU/ml in the presence of *C. jejuni* at high concentrations of 10^6 CFU/ml and 10^7 CFU/ml (Table 8). However, *C. jejuni* at a concentration of 10^4 CFU/ml was detectable only with a low concentration of *S.* Typhimurium (10^2 CFU/ml). Moreover, the concentrations of *C. jejuni* below 10^4 CFU/ml were indeterminate in the presence of any tested *S.* Typhimurium concentrations (10^2 - 10^7 CFU/ml) in chicken rinse. Apart from that, the detection of *S.* Typhimurium at concentrations of 10^3 - 10^6 CFU/ml was not interfered by the simultaneous detection of *C. jejuni* at concentrations of 10^4 - 10^7 CFU/ml in artificially contaminated chicken rinse by the multiplex STN-rtPCR assay (Table 8).

Table 8. Evaluation of the developed multiplex single tube nested real-time PCR assay with chicken rinse inoculated with *S.* Typhimurium and *C. jejuni*. [a]

Bacterial concentration (CFU/ml)		Ct value	
S. Typhimurium	*C. jejuni*	*S.* Typhimurium	*C. jejuni*
10^6	10^7	10.83[b] ± 0.18[c]	13.87 ± 0.52
10^6	10^6	9.43 ± 0.33	20.12 ± 0.74
10^6	10^5	8.40 ± 0.15	24.92 ± 0.35
10^6	10^4	8.29 ± 0.35	-[d]
10^5	10^7	12.77 ± 0.28	8.78 ± 0.07
10^5	10^6	10.23 ± 0.28	11.92 ± 0.39
10^5	10^5	10.22 ± 0.02	25.39 ± 1.01
10^5	10^4	10.26 ± 0.40	-
10^4	10^7	16.70 ± 0.78	8.81 ± 0.51
10^4	10^6	16.28 ± 0.68	11.70 ± 0.79
10^4	10^5	15.83 ± 0.86	19.18 ± 1.90
10^4	10^4	15.85 ± 0.87	-
10^3	10^7	23.52 ± 0.34	9.04 ± 0.08
10^3	10^6	22.52 ± 0.75	11.35 ± 0.56
10^3	10^5	22.00 ± 0.49	19.66 ± 0.58
10^3	10^4	21.48 ± 0.85	-
10^2	10^7	-	8.76 ± 0.47
10^2	10^6	-	11.10 ± 0.76
10^2	10^5	28.23 ± 0.10	15.54 ± 0.24
10^2	10^4	28.37 ± 0.16	24.46 ± 0.36

[a] No amplicon was observed from uninoculated chicken meat rinse samples.
[b] Ct values are averages from three separate experiments.
[c] Standard deviation.
[d] No fluorescence signal detected after 30 cycles was shown as -.

4.4 Discussion

Food contamination caused by pathogens has attracted increasing attention worldwide, which not only causes billions of dollars in losses but also poses severe threats to human health. *Salmonella* and *Campylobacter jejuni* are two most common causes of foodborne disease. They have a significant economic impact on the poultry industry as various domesticated and wild birds are natural reservoirs of these pathogenic bacteria. Conventional culture-based detection methods for these pathogens often require several days to obtain results and do not always supply information rapidly enough to allow appropriate actions needed to protect the public. Therefore, this study developed a sensitive multiplex STN-rtPCR assay with TaqMan probes for simultaneous detection of *Salmonella* and *C. jejuni* and investigated the efficacy of the established assay using chicken rinse inoculated with the target pathogens.

Nested primers and probes were designed to target the *inv*A gene of *Salmonella* spp. and the *hip*O gene of *C. jejuni*. The optimal annealing temperature for the two pairs of outer primers was 65ºC which was 10ºC higher than that for the two pairs of inner primers. Therefore, the two rounds of PCR were able to be separately processed in a single closed tube to avoid potential cross-contamination of amplicons associated with nested PCR. Tests were first performed to set up the uniplex STN-PCR for *Salmonella* and *C. jejuni* individually. The optimal concentrations of primers identified in the multiplex STN-PCR assay were validated by analyzing the amplification products via gel electrophoresis, which turned out that the expected two amplicons with comparable intensity were clearly visible in the gel when 40 pmol *Salmonella* inner primers, 20 pmol *C. jejuni* inner primers, 0.1 pmol *Salmonella* and 0.1 pmol *C. jejuni* outer primers were used. The specificity test of the established multiplex STN-PCR assay was performed. All tested strains of *Salmonella* spp. and *C. jejuni* yielded expected amplicons of 226 bp and 173 bp, respectively, while no amplification products were observed with other nine

bacteria strains being tested.

The sensitivity of the multiplex STN-rtPCR assay was evaluated after the concentrations of TaqMan probes were optimized. This study demonstrated that the novel multiplex STN-rtPCR assay was able to simultaneously detect 1 pg of *S.* Typhimurium and *C. jejuni* DNA using 10 pmol of specific probe for *Salmonella* and 5 pmol of specific probe for *C. jejuni* in the reaction. Alves *et al*. (2016) reported a detection limit of 100 pg DNA for *Salmonella* and *C. jejuni* using a multiplex real-time PCR assay they developed. The multiplex STN-rtPCR assay in this study was 100-fold more sensitive than conventional multiplex rtPCR. This is consistent with previous studies for the detection of plant viruses (Llop *et al*., 2000; Dey *et al*., 2012; Hamim *et al*., 2018), which also illustrated that the sensitivity of STN-PCR was at least 100-fold higher than conventional PCR.

Multivariable analysis disclosed that the risk for *Salmonella* and *C. jejuni* to contaminate poultry carcasses increased with the processing of chicken carcasses in the slaughterhouse (Arsenault *et al*., 2007; EFSA, 2010; Seliwiorstow *et al*., 2016). The applicability of the established assay was evaluated for simultaneous detection of *Salmonella* and *C. jejuni* in artificially contaminated chicken rinse. The detection limit of the multiplex STN-rtPCR assay was 10^2 CFU/ml for either target in chicken rinse. Additionally, the developed assay exhibited a comparable efficiency to the uniplex STN-rtPCR assay for detecting *Salmonella* of 10^3 - 10^7 CFU/ml even in the presence of high concentrations of *C. jejuni*.

In 2007, Wolffs *et al*. (2007) separated target organisms form background microflora by floating them in a discontinuous density gradient. Then, a real-time multiplex PCR assay with hybridization probes was confirmed to be able to detect *Campylobacter* and *Salmonella* at levels as low as 7.1×10^5 CFU/ml and 3.0×10^3 CFU/ml, respectively, in spiked chicken skin rinse.

Besides, Alves *et al*. (2012) indicated that the sensitivity of multiplex PCR with spiked chicken rinse was 10^2 CFU/ml for *C. jejuni* after 24 h of selective enrichment and 1CFU/ml for *Salmonella* Enteritidis after 24 h of nonselective enrichment. Years later, Alves *et al*. (2016) displayed a multiplex TaqMan probe-based real-time PCR assay including an internal amplification control for simultaneous detection of *Campylobacter* spp. and *Salmonella* spp. in chicken rinse. A rude DNA extraction method was applied. They demonstrated that 0.1 ng of both target DNA was detectable in the reaction. A detection limit of 10^3 CFU of *Campylobacter* spp. and 10^6 CFU of *Salmonella* spp. per milliliter of artificially contaminated chicken rinse was confirmed without enrichment, while 1 CFU of either target per milliliter was identifiable after non-selective enrichment for 24 hours. Moreover, Yang *et al*. (2014) revealed a multiplex real-time PCR with minimum detection limits of 10^3 organisms/g for both *Campylobacter* spp. and *Salmonella enterica* in lamb feces. It is worth noting that the multiplex STN-rtPCR assay developed in this study allowed for ultra-sensitive detection of *Salmonella* spp. and *C. jejuni* in chicken rinse within 4 hours. This assay can also eliminate the potential of amplicon cross-contamination associated with two-step nested PCR and reduce testing time and the amount of reagents required by uniplex PCR method. This novel assay could potentially facilitate clinical studies of foodborne illnesses caused by pathogenic bacteria and help improve food safety systems by detecting low levels of *Salmonella* spp. and *C. jejuni* in chicken earlier.

There are several limitations of the multiplex STN-rtPCR assay in this study, such as the requirement for TaqMan probes and commercial kit for DNA extraction from food samples, which increase the cost for analysis. Besides, the thermal cycler is needed for completing all reactions, making this assay only practicable in the laboratory. Therefore, this assay could be modified by analyzing the melting curves of target amplicons in the presence of a fluorescent

intercalating dye such as SYBR Green or SYT09 (Agrimonti *et al*., 2018; Singh and Mustapha, 2014). Another improvement of this assay could be using portable devices which might allow on-site field applications. The recombinase polymerase amplification (RPA) is an alternative for simple and fast diagnose of pathogenic bacterial in the remote areas (Daher *et al*., 2016). It might be possible to develop a simple noninstrumented nested RPA assay for point-of-care diagnostic with well-designed RPA primers and probes.

Future studies may expand this protocol to other pathogens and other varieties of food. In advance, improvement is necessary for the sample preparation method. It needs to be mentioned that the choice of DNA extraction method is critical for effective detection of target pathogens. In this study, we found that the commercial DNeasy kit was preferable than PrepMan Ultra reagent in order to extract DNA from chicken rinse samples. It was observed that the DNA samples extracted using PrepMan Ultra reagent could affect the amplification efficiency of the reaction. This phenomenon can be explained by the fact that organic and inorganic substances in food such as fat, salts, proteins and polysaccharides can interact with PCR reagents and further inhibit PCR amplification. Therefore, a suitable extraction reagent should be considered toward different food classes. Alternatively, concentration of target pathogens could be included prior to DNA extraction in order to improve the sensitivity of detection methods. Nanoparticle-based immunomagnetic separation (IMS) has been introduced as a promising approach to isolating target bacterial cells form complicated food matrices (Chen and Park, 2018), which might be helpful to increase the sensitivity of detection. Moreover, a comparison of the developed multiplex STN-rtPCR assay with conventional culture methods with naturally contaminated food samples would further evaluate its sensitivity and specificity.

Chapter 5

Conclusions

A single tube nested PCR (STN-PCR) assay was developed for rapid and sensitive the detection of *Campylobacter jejuni*. Outer primers and inner primers were designed based on the hippuricase (*hip*O) gene of *C. jejuni*. The annealing temperatures for the outer and inner primers were optimized to be 65ºC and 55ºC, respectively. In addition, the optimal concentrations of outer and inner primers were 0.1 pmol and 40 pmol, respectively. The specificity of the established STN-PCR assay was confirmed with thirteen bacterial strains. The detection limit was determined to be 10 *C. jejuni* DNA copies, which was 100 times more sensitive than conventional PCR with inner primers. Furthermore, the single tube nested real-time PCR (STN-rtPCR) assay using SYBR Green was successfully applied to detect as low as 36 CFU/ml of *C. jejuni* in artificially contaminated ground chicken homogenate without enrichment. After 24 h of enrichment, the ground chicken homogenate with an initial inoculum of 0.1 CFU/g *C. jejuni* yielded positive results by STN-rtPCR, whereas the sample required a 48 h enrichment to be tested positive by both standard culture-based method and conventional rtPCR. Moreover, compared with 48 h of enrichment needed by the culture method, 6 h of enrichment was adequate for the STN-rtPCR assay to identify 1 CFU/g *C. jejuni* in ground chicken homogenate. These results illustrate that the developed STN-rtPCR assay provides an effective tool for rapid detection and accurate quantification of *C. jejuni* in ground chicken.

A multiplex single tube nested real-time PCR (multiplex STN-rtPCR) assay was developed for simultaneous identification of *Salmonella* spp. and *C. jejuni*. Initially, nested primers and TaqMan probes were designed to target the *inv*A gene of *Salmonella* spp. and the *hip*O gene of *C. jejuni*. With the established amplification system, all tested strains of *Salmonella* spp. and *C.*

jejuni yielded expected amplicons of 226 bp and 173 bp, respectively, while no amplification products were observed with other tested bacterial strains. The sensitivity of this novel multiplex STN-rtPCR assay was determined to be 1 pg/µl of *Salmonella* Typhimurium and *C. jejuni* DNA. Furthermore, the detection limit of the developed assay was 10^2 CFU/ml of *S*. Typhimurium or *C. jejuni* in artificially contaminated chicken rinse. It also exhibited a comparable efficiency for co-amplifying DNA extracted from 10^7 to 10^2 CFU/ml of *Salmonella* and *C. jejuni* in chicken rinse.

A major limitation of the STN-rtPCR assay is the requirement of thermal cycler which makes this assay unsuitable for field application. Thus, other portable approaches such as RPA could be taken into consideration to achieve the purpose of point-of-care diagnose. Besides, the TaqMan probes added extra cost for detection, which could be reduced by analyzing melting curves of amplicons using fluorescence dyes like SYBR Green or SYTO9 (Agrimonti *et al*., 2018; Singh and Mustapha, 2014).

In summary, the STN-rtPCR assays developed in this study were demonstrated to be promising tools with superior specificity and high sensitivity for rapid detection of *Salmonella* spp. and *C. jejuni* in chicken products. Further studies may scale up this protocol to other foodborne pathogens and more varieties of food, while appropriate food preparation methods should be optimized preliminarily. In addition, target pathogens in the sample may be concentrated prior to the STN-rtPCR assay to further improve the sensitivity of detection. Nonetheless, the advantages of these novel STN-rtPCR assays appear to be well worth the effort invested.

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